Enhancement of Hydrocarbon Degradation using Biosurfactants from *Rhodococcus* species in a Membrane Reactor System

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Abstract

Bacterial degradation of hydrocarbons has achieved greater prominence after the discovery of organisms that have been reported to degrade a host of recalcitrant compounds. The ability of bacteria to degrade hydrocarbons is a viable alternative to other energy intensive clean up options. One of these degradative pathways is through the production of biosurfactants, which have exhibited greater advantages than their chemical counterparts. In this work, two strains of the robust *Rhodococcus* genus namely *Rhodococcus opacus* and *Rhodococcus ruber* have been tested for their ability to degrade n-hexadecane. In order to elicit the maximum production of surfactants, the optimisation of the growth medium with respect to nitrogen source, temperature and rotation speed was carried out. The Phenol-Sulphuric acid assay was employed to quantify the amount of surfactant produced. Biosurfactant efficacy was then determined by measuring the emulsification activity, adhesion to hydrocarbons and reduction in surface tension. Two different mixed-culture reactors were utilized whereby one involved use of a conventional 3L automated batch reactor and another where a specially designed membrane reactor, in which by the use of membrane partitions, enables the two strains of bacteria to be physically separated. From the experiments carried out there is clear indication that using a membrane reactor would be better suited for mixed culture reactor systems. Mixed culture membrane reactors are promising in the field of bioremediation.

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Chapter 1: Introduction

This chapter reviews the relevant background literature that has been carried out in the field of bioremediation and other related work that has been carried out in this project. All sections focus mainly on the direct implications of the research carried out in each field relevant to the experiment work that has been carried out.

1.1 Bioremediation

A majority of organic and inorganic chemicals are subject to enzymatic degradation by various microorganisms. Most of the environmental pollutants present in the environment can be included among these chemicals and the enzymatic degradation of these pollutants can be termed as biodegradation (Crawford, 1996). However the term biodegradation encompasses a wide variety of techniques, which have varied outcomes. There may be many processes involved before a single contaminant may be completely degraded.

Bioremediation is the productive use of these biodegradation processes to eliminate the various industrial pollutants that eventually find their way into the environment (Crawford, 1996). Bioremediation processes could heavily depend on a number of factors such as presence of toxic agents, temperature, nutrients, bioavailability of compounds and oxygen limitations (in aerobic processes). The use of bioremediation has recently attracted a lot of attention due to the recent developments in pollutant classifications and also discovery of specialized microorganisms that have the capability

to degrade many of these pollutants. One of the main drivers that stimulate research into bioremediation is economics. It is estimated that the cost of bio-treatment of biodegradable contaminants in soils ranges from $40 - $100 per cubic yard, as compared to high costs of around $250 - $800 per cubic yard incurred for incineration and $150 - $250 per yard for land filling (Levin and Gealt, 1993). Some of the advantages of using bioremediation include:

- Simultaneously treat subsurface soil and water.
- Be completed in less time than traditional pump-and-treat operations.
- Eliminate long-term liability of pollutant accumulation.
- Result in complete mineralization of the contaminants.
- Be conducted under mild operating conditions.
- Easily be combined in a treatment train.
- Be completed with little or minimal stress on environmental and ecological systems.

(Cutright, 2005)

Bioremediation encompasses two main types of processes. Ex-situ bioremediation where the contaminated material is physically relocated to be treated and in-situ bioremediation, which involves treatment of contaminants in their place of origin. Some of the technologies that are commonly used are:

1. Land farming: This is a solid-phase treatment system mainly used for contaminated soils where they are inoculated with specialist organisms and tilled continuously until the required level of contaminant degradation is achieved.
2. Composting: This technique is similar to the land farming method but this is aerobic thermophillic reaction between the contaminated soils with the bulking

agent. Composting could be done in open static piles or in the case of larger sites they are aerated.

3. Bioreactors: It is an example of ex-situ bioremediation where the contaminants are removed from the site and are mixed along with specialist organisms in a closed vessel or bioreactor. This method is usually used for slurries or liquid wastes.
4. Bioventing: This is a process of artificially pumping oxygen or air through the land bed. The supplied oxygen would stimulate the growth of naturally occurring microorganisms and help in the degradation.
5. Biofilters: These are similar to ordinary filters but consist of microbial stripping columns, which are then used to treat air pollutants.
6. Bioaugmentation: This is a very common method, which basically is the addition of specialist organisms that have known degrading capabilities. Bioaugmentation could be performed either in-situ or ex-situ.
7. Biostimulation: Very similar in concept to the bioventing technique, which involves the pumping of the required nutrients into the contaminated sites to induce the growth of the naturally occurring microbial population.

Biostimulation is a technique that relies on increasing the activity of the indigenous bacteria by the addition of factors that stimulate their growth. These could enhance the limiting factors such as nutrients, air, mobility etc. Treatments such as this could be more effective since the indigenous bacteria are better adapted to survive the soil environment that requires treatment (Rahman *et al*., 2003).

As opposed to biostimulation another approach that is currently under study is bioaugmentation. This involves the introduction of an effective bacterial isolate/or consortium of bacteria with known xenobiotic degradation capabilities into

contaminated sites (Gentry *et al*., 2004). In the use of bioaugmentation there are two methods that could be employed namely in-situ and ex-situ. Ex-situ bioaugmentation involves the transfer of contaminated soil to various specialized treatment facilities, which need to be constructed. As compared to ex-situ methods, in-situ approach is relatively cheaper to pursue and cost effective in remediation of soil and ground water (Bouwer *et al*., 1994). One very pronounced problem of this method is the capability of the exogenously added bacterial strains to survive in a foreign environment. This process of acquiring required tolerance could take prohibitively long time. In a study carried out by Ueno *et al*. (2007) found that their test organism *P*. *Aeruginosa* strain Wat G was completely unable to survive when administered to unsterilized paddy soil that had been previously artificially contaminated with diesel oil, whereas this was not the case when grown in sterilized soil. This would not suit the need to identify bacteria that can be used in bioaugmentation.

As discussed above the effectiveness of bioaugmentation and biostimulation in bioremediation of hydrocarbon contamination is questionable and in many cases has been proven not to work. Another field of interest has been the application of immobilized cells in bioremediation activities. Immobilization offers various advantages over conventional liquid cultures in bioaugmentation. There is evidence suggesting that the immobilization matrix acts as the bulking agent in contaminated soil (Cunningham *et al*., 2004) and offer protection from environmental factors such as pH, temperature and toxic compounds. A study by Lozinsky *et al*. (2008) obtained promising results that indicated a strong possibility of developing a wide range of cheap biodegradable cellulose-containing carriers.

Immobilization could also be effectively used when there is a requirement to use more than one bacterial species. In the treatment of model petroleum contaminated

water, it was found that a bacterial mix of *Rhodococcus opacus* and *Rhodococcus ruber* immobilized onto straw dust effectively degraded nearly 80% of certain hydrocarbons within 3 weeks. The use of continuous bioreactors would enable higher volume of waste treatment and in such a scenario immobilization could be the only way to preserve the bacteria and preventing decline in active biomass (Kuyukina *et al*., 2009)

Considering the various disadvantages and uncertainties of bioaugmentation and biostimulation there has been considerable scientific interest in yet another new approach. Cameotra and Bollag (2003) demonstrated that a biosurfactant produced by a bacterium enhanced the remediation efficiency by solubilising the target polyaromatic hydrocarbon and hence enabled better utilization by the indigenous organism. The use of such a method would offset the disadvantages faced with the other conventional methods. In a promising study carried out by Das *et al*. (2008), a marine biosurfactant producing bacteria aided in the solubilisation of anthracene. When the bacterium was grown in mineral media with anthracene as sole carbon source no growth was observed but when supplemented with glycerol, greater biosurfactant activity was observed with decreasing anthracene concentration thus proving that the biosurfactant produced aided in the anthracene uptake.

Currently a lot of work is done in order to find new organisms with the capacity to degrade many of the industrial pollutants such as PCB's, heavy metals, dyes and similar industrial effluents. Among these chemicals, hydrocarbons form some of the most abundant environmental pollutants. In a study of 242 accidents with storage tanks occurring over 40 years, it was found that 74% of accidents had taken place in petroleum refineries, oil terminals or storage facilities (Chan and Lin, 2006). Petroleum products contain several individual hydrocarbons which are indigenous to their location and mainly constitute saturates (n- and branched chain alkanes and cycloparaffins) and

aromatics (mono-, di, and polynuclear aromatic compounds containing alkyl side chains) (Speight, 1991). This being said, it has been found that hydrocarbons constitute one of the major environmental pollutant and they have been found to be widely spread in several polluted environments (Casellas *et al*., 1995; Fernandez *et al*., 1992).

One of the major routes of degradative pathway for hydrocarbons is degradation by the microbes present at the site of contamination. This can be greatly hampered by the fact that the rates of biodegradation in the environment are limited due to the hydrophobicity and low water solubility, which makes the hydrocarbons non-available (Providenti M.A *et al*., 1995; K.S.M Rahman *et al*., 2001, S. Kuyukina *et al*., 2004). This is where the possibility of utilization of biosurfactants has been investigated. Biosurfactants can greatly enhance the bioavailability of slightly soluble organic compounds such as hydrocarbons. This is achieved by a variety of methods such as:

a) Increasing the surface area of the substrate by dispersion
b) Improving the microbial adhesion to the substrate

(Rahman *et al*., 2003; Manoj Kumar *et al*., 2006)

In order to optimise the process and in order to find the right organisms, a basic understanding of the factors that affect the rate of biodegradation is required. Some of the factors affecting the rate of bioremediation are summarized below. Although the list is not entirely exhaustive these are the important factors that have an effect on the bioremediation: (Boopathy, 2000)

1. Microbial factors: Microbial limitations could include growth limitations due to critical biomass concentration, mutation that could occur in the organism and the accumulation of toxins. These factors can be analysed with sufficient field trials.

Other factor could include the microbial interactions such as competition for raw materials, and predation among microbial populations.

2. Environmental factors: These always have an effect on the rate of bioremediation. Microbial growth could be inhibited by the depletion of the required nutrients and also by other inhibitory environmental conditions such as pH, salinity and temperature.
3. Substrate: This is one of the most important limiting factors. There have been cases where the chemical structure of the contaminant prevents it from being used as a substrate and the chemical could be toxic to the microbial population. In some cases the substrate could be insoluble or the substrate concentration too low to be effectively utilized or to support microbial growth. Another factor could be the problem of co-metabolism. In any open environmental system it is not possible to maintain the presence of only one nutrient source. It is highly likely that the organisms may use an alternative source of carbon than the intended contaminant.
4. Mass transfer limitations: In a biological process mass transfer of nutrients is of dire importance. The site of degradation in case of in-situ bioremediation should not have any oxygen diffusion or solubility limitations. If the sand is of poor porosity then this would create severe mass transfer limitation.

There has been research carried out in investigating the action of microbes on a wide variety of organic compounds that also include in-situ treatment of petroleum contamination by bioventing (Raymond, 1974), munitions degradation like 2,4,6-trinitrotoluene (TNT) and hexahydro- 1,3,5-trinitro- 1,3,5- triazine (RDX) (Kaake *et al*., 1992; Funk *et al*., 1993). Considerable work has been done on bioremediation of hydrocarbons that form a majority of the organic pollutants. Reviews on the aspects of hydrocarbon remediation include Leahy & Colwel (1990), Atlas (1991) and Rosenberg

(1993). Bioremediation has also effectively addressed the problem of polychlorinated biphenyls (PCBs). The ill effects of PCBs were noticed in the 1960s in Japan and Taiwan. With the advent of tools of genetic engineering, PCB degradation was more easily achieved due to greater metabolic activity of microorganisms on PCBs (Furukawa *et al*., 1989).

The other important aspects of bioremediation are further discussed in Chapter 1, Section 1.3.4 with more emphasis on the role of biosurfactants in bioremediation.

1.2 The Genus *Rhodococcus*:

1.2.1 Introduction:

The recent interest shown towards the genus *Rhodococcus* has been kindled by the species ability to degrade a wide variety of chemicals and also the capability to synthesize several products of commercial interest such as surfactants, flocculants, amides and polymers. Rhodococci are described as aerobic, Gram-positive, non-motile, mycolate-containing, nocardioform actinomycetes (Goodfellow, 1989). There have been a lot of changes in the taxonomical classification of the various species that belong to the genus *Rhodococcus* (Christofi *et al*., 1998). Currently after all the classifications have been taken into account there are 12 established *Rhodococcus* species. The *Rhodococcus* genus shares the capability to degrade a vast majority of substrates with the other distantly related species Pseudomonas. Dagley (1984) states "On thermodynamic grounds, no organic compound can be excluded from serving as a possible energy source for aerobic micro-organisms" This is further supported in the case of the genus *Rhodococcus*.

The genus *Rhodococcus* are being considered in this research due to their novel enzymatic capabilities, which confer in them the capability to degrade a wide variety of xenobiotic compounds (Finnerty, 1992). *Rhodococcus* can degrade a wide range of chemical compounds such as phenols, aromatic acids, halogenated phenols, halogenated alkanes, substituted benzenes, anilines and quinolines. In the review by Geize *et al*. (2004) titled "Harnessing the catabolic diversity of rhodococci for environmental and biotechnological applications", the authors demonstrated that there is extensive literature to show that *Rhodococcus* strains harbour a wide variety of linear plasmids. These are capable of degrading compounds that could be considered difficult in respect

to their recalcitrant nature and even potentially harmful to other microbes. Some very recalcitrant chemicals such as thiocarbamate, s-triazine herbicides and 2-mercaptobenzothiazole can be catabolised by *Rhodococcus* strains (Larkin *et al*., 2005). *Rhodococcus* strains have also been very specifically used to remove sulphur from coal and oil after hydrosulphurisation, which releases recalcitrant s-heterocyclic compounds. *Rhodococcus* strains are the only bacteria effective in this respect. This has achieved considerable importance, since de-sulphurisation is a critical process in the fossil fuel industry as it increases the calorific value of the fuel as well as increasing environmental safety (Geize *et al*., 2004).

Apart from the presence of a diverse range of plasmids that confer biodegradability, the *Rhodococcus* genus also have physiological attributes that help in its biodegradation abilities. The wide catabolic diversity of the *Rhodococcus* genus can be attributed to genetic and physiological adaptations. In general rhodococci are known to survive high concentrations of hydrocarbons and the natural ability to survive in a range of water miscible solvents (ethanol, methanol, dimethylformamide, butanol up to 50% v/v) and water immiscible solvents such as dodecane and toluene. Another ability is the production of biosurfactants in presence of water immiscible solvents and presence of mycolic acids in their cell walls that allow attachment to oil-water interfaces. Apart from their physiological abilities the genetic capabilities are the primary reason behind their robustness. The presence of large genomes that rely on acquisition of genes to employ as recombination substrates and the presence of linear plasmids make the *Rhodococcus* genus well suited in the long-term evolutionary strategy (Larking *et al*., 2005).

Rhodococcus are specifically known to produce a class of biosurfactants known as glycolipids, which are usually associated with trehalose bound to the primary structure of the surfactant (Philp and Lang, 1998). In the study carried out by Zaragoza *et al*. (2009) they have demonstrated that in spite of being a weaker surfactant as compared to widely used surfactants such as Surfactin produced by *Bacillus subtilis*, trehalose-glycolipids are very effective at permeabilizing phospholipid membranes. This could be advantageous when used in a bacterial consortium. The presence of such a surfactant in the medium could indirectly enhance the effectiveness of other bacteria specifically selected for their robust bioremediation capabilities.

The genus *Rhodococcus* has well documented hydrocarbon degradative capabilities including gaseous forms of hydrocarbons. Among the various genetic metabolisms associated with the degradative abilities, one that is of commercial importance is the ability to produced surfactants. The degradative capabilities are linked to biodegradation in three ways:

1. The presence of cellular surfactants such as mycolic acids, causes fixation of the microbial cells to the oil-water interface in bi-phasic systems. This has varied applications in commercial fermentations as well as environmental applications.
2. Surfactants reduce the interfacial tension between the two immiscible phases allowing easier migration of the hydrocarbons into the cell wall.
3. Extra cellular surfactants can easily disperse the hydrophobic compounds thus increasing the surface area of microbial attack.

According to Ajay *et al*. (2007) the rate of biodegradation of a contaminant depends on its bioavailability to the metabolising organism. Hence the presence of surfactants

and emulsifiers would increase the pseudo-availability of the contaminants, which are generally known to exhibit low water solubility.

Due to the wide catabolic diversity of the genus *Rhodococcus*, this will be further investigated in this research work. The industrial and environmental applications of *Rhodococcus* are further discussed below.

1.2.2 Applications:

The various species of the genus *Rhodococcus* produce surfactants (surface active lipids) in the presence of alkanes. The main role of these glycolipids is to ensure the uptake of the hydrophobic alkanes as growth substrates (Lang & Philp, 1998). Surfactants are a group of commercially important chemicals and the demand for them are expected to increase many fold in coming years (Desai & Banat, 1997). However, biosurfactants are yet to achieve significant market penetration.

This scenario is changing with increased understanding of the genetics behind the various species of *Rhodococcus*, which helps in the understanding of the metabolism behind surfactant production (Finnerty, 1992; Wever *et al*., 1997; Christofi *et al*., 1998; Geize, 2004; Larkin *et al*., 2005). Coupled with this is the increasing concern over the environmental acceptability of chemical surfactants that are currently in use. Rhodococcal surfactants compete with synthetic surfactants in their ability to reduce interfacial tension and have sufficiently low critical micelle concentration (CMC). Several members of the genus *Rhodococcus* have been used to produce surfactants that match the capabilities of their chemical counterparts (Christofi & Ivshina, 2002; Christofi *et al*., 2002; Shao *et al*., 2006).

Biological transformations have received greater interest over chemical methods. The use of microbial transformations essentially means that one of the enzymes produced by the organism helps in conversion of the substrate into another product. Occasionally these enzymes also have the capability to convert similarly structured compounds and hence this pave the way for microbial transformations (Kobayashi *et al*., 2005). One of the main industrial application of *Rhodococcus* is its ability to produce nitrile hydratase (NHase) and nitralase, which help in the degradation of nitrile compounds. Polyacrylamides are a very widely used group of chemicals, which find a place in almost every field of chemical processes and industries. Their need touches several thousand tonnes per year (Hughes *et al*., 1998). On the other hand they are extremely toxic in nature and inadvertently find their way into the environment. For example, acrylonitrile is toxic and a mutagenic agent and acrylamide, a precursor to polymer manufacture is a known neurotoxin. Hence the application of *Rhodococcus* is twofold. One is the environmental clean-up activities that could lead to the production of a commercially important chemical precursor and another is that it could be used in-situ to extract surfactants. In the review by Hughes *et al* (1998) it is evident that considerable amount of commercialization of the Rhodococcal enzymes have been achieved. Most work has been done on *Rhodococcus rhodochrous* species but it is evident that other members of the genus are likely to have this capability.

Rhodococcus strains have also been used in hydrocarbon degradation for use in environmental applications. Harayana *et al*. (2000) found that a mucoidal strain of *Rhodococcus rhodochrous* produced an extra-cellular polysaccharide (EPS) that enabled the organism to survive in concentrations of up to 10% (v/v) n-hexadecane. After isolation, the EPS was found to also confer the hydrocarbon tolerance to other strains as

well. In another study, *Rhodococcus* erythropolis was found to be very efficient in hydrocarbon removal from oily sludge (Pireira *et al*., 2006).

Several species of the genus *Rhodococcus* have been reported to produce trehalolipids that have remarkable emulsifying activity (Calvo *et al*., 2009). It has been clearly demonstrated that the presence of biosurfactants whether by direct addition or by the presence of exogenously added bacterial strains clearly shorten the degradation and adaptation time of the microbes (Kosaric, 2001). Also it was demonstrated that the presence of trehalose lipid biosurfactants in mud cakes that had been contaminated with Arabian light crude oil resulted in the elimination of polycyclic aromatics and results in complete degradation.

Another aspect for consideration is the genus capability for high solvent tolerance. Solvent tolerance is conferred to the members of the *Rhodococcus* genus as discussed previously. These tolerances are all regulated by the wide range of linear plasmids. Some of the physiological changes bacterial organisms undergo in the presence of organic solvents are:

1. Changes to the cell wall and membrane composition
2. Change in the protein content of the membrane
3. Modification in the properties of the cell wall
4. Key changes in the cell morphology
5. Change in the enzymatic pathways utilized to assimilate the solvent

(Weber & Bont, 1996; Meinhardt *et al*., 2007)

In recent years a lot of emphasis has been placed on whole biotransformation as it is believed to be superior to enzyme catalysed reactions in certain aspects (Leon *et al*., 1998). In traditional aqueous phase reactions, product recovery from the aqueous phase becomes expensive to carry out. It is possible that if an organic solvent phase is used this could be minimised as the product would be preferentially absorbed into the solvent phase while the accumulation of toxic substances would be in the aqueous phase. In such a case, only bacteria being able to survive in the presence of toxic solvents can be used. This is a great advantage of the genus *Rhodococcus* that can naturally grow on or in the presence of various organic solvents. According to Meinhardt *et al*. (2007) it is estimated that biotechnological production of niche chemicals such as pharmaceutical products would soon be able to compete economically with chemically synthesized products by the use of solvent tolerant bacteria.

It is known that the majority of the cost factors involved in the production process of an industrial product are incurred in the downstream processing of the substance. Hence this process could be an important step towards the production of various chemicals through the biotechnological process, as a two-phase system would considerably reduce the requirement of downstream processes. This would involve the use of a water immiscible solvent as mentioned above. In such a system any beneficial secondary metabolites produced would be preferentially absorbed into the solvent phase, which would directly have an impact on the downstream processing that would be required to extract the metabolites. This would in turn have an effect on the production costs.

One of the aims of this work is to adapt the species *Rhodococcus opacus* to grow in higher concentrations of glycerol, methanol and to a certain extent a crude derived form of glycerol. The crude form of glycerol was derived as a by-product of biodiesel production and was provided by Neil Hollow as part of another PhD project. This work was carried out in order to access the impact it would have on the growth of the bacteria in the presence any impurities that might be produced during the production of biodiesel.

1.3 Biosurfactants:

1.3.1 Introduction:

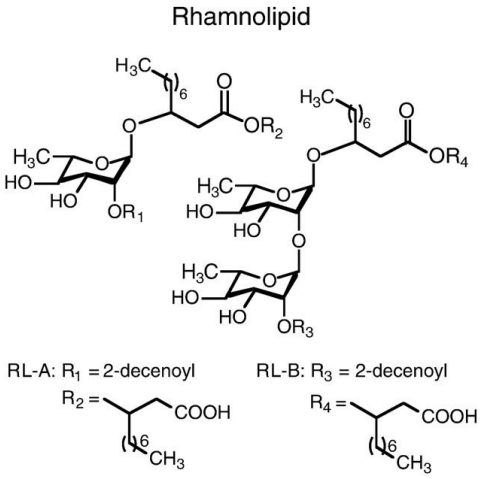
Very broadly a surfactant can be defined as "A surface active amphiphile that aggregates (self-assembles) in water or other solvent to from various micro-structures such as micelles or bilayers" (Lange, 1999). Biosurfactants are a class of chemicals that are produced by microorganisms (Banat, 1994). Carla *et al*. (2009) demonstrated that certain bacteria are capable of adapting their physiological properties in order to be able to metabolize hydrocarbons. This is usually coupled with the ability to produce biosurfactants that aid in the uptake of metabolites that have relatively low water solubility. Currently a lot of research is being carried out on microbial surfactants and *Rhodococcus* is of interest due to the wide range of surfactants that the various members of the genus produce (Cooper, 1986; Cooper & Goldenberg, 1987; Ron & Rosenberg, 2001; Tyagi & Gautham, 2006).

Unlike most classifications for chemical surfactants that are based on the nature of their polar grouping, biosurfactants are generally characterized by their chemical composition and their microbial origin. Generally their typical composition would

include a hydrophilic moiety composed of amino acids and a hydrophobic moiety that would be made up of unsaturated, saturated, or fatty acids. The fatty acids that form the side chains in a surfactant molecule confer different attributes to the nature of the biosurfactant. For example in the case of trehalose lipids as shown in illustration 1.b below, the side chain molecules are composed of Mycolic acids. Mycolic acids are long chained branched hydroxy fatty acids and differ in size, structure of mycolic acid, number of carbon atom and the degree of unsaturation depending on the organisms they are extracted from (Tyagi & Gautham, 2006).

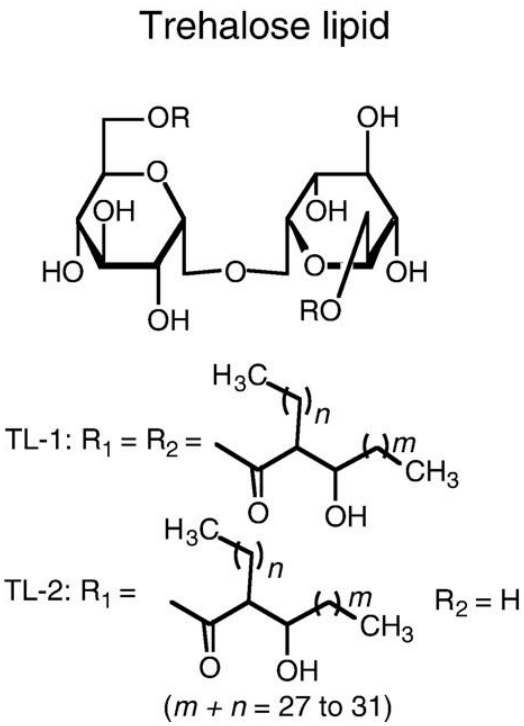
These varieties of surfactants are classified based on their chemical composition and structure. Broadly they are:

1. Glycolipids
    a. Rhamnolipids



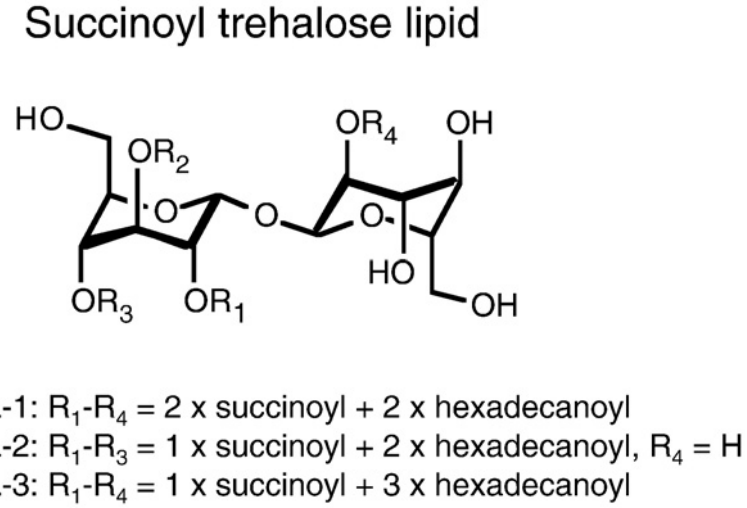
Chemical structures for Rhamnolipids, Kitamoto *et al*. (2009)

b. Trehalolipids



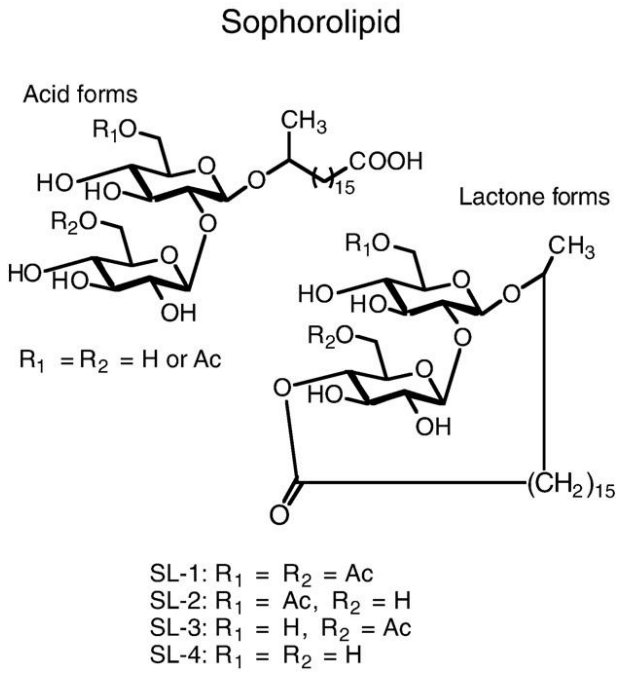
Chemical structures for Trehalolipids, Kitamoto *et al*. (2009)

In the above illustration as detailed above surfactants that are clasiffied as trehalose lipids are composed of hydrophillic trehalose molecules and hydrophobic mycolic acid chains which are usually within m+n ranging from 27-31 molecules in length.



Chemical structures for Succinoyl trehalose lipid, Kitamoto *et al*. (2009)

c. Sophorolipids



Chemical structures for Sophorolipids, Kitamoto *et al*. (2009)

2. Lipopeptide and Lipoprotein
3. Fatty acids, Phospholipids and Neutral lipids
4. Polymeric biosurfactants
5. Particulate biosurfactants

(Tyagi & Gautham, 2006)

Another method of biosurfactant classification is carried out based on their molecular weights, which could be linked to the length of the fatty acid side chains that are attached to the surfactant molecule:

1. Low molecular weight surfactants: These are surfactant molecules that are primarily involved in the lowering of surface tension of water and typically have short straight chained fatty acids.

2. High molecular weight surfactants: These help in microbial adhesion to surfaces and typically composed of long chained branched fatty acid molecules attached to the hydrophilic moiety of the biosurfactant molecule.

   (Kitamoto *et al*., 2009)

According to Christofi and Ivshina (2002) the above classification based on molecular weights should be considered to be part of the classification that is based on the chemical structure and microbial origin rather than a separate form of classification as the property of molecular weights is relative to the organism from which the biosurfactant has been extracted.

To understand the role of surfactants it is necessary to understand the genetics behind their production. Hua Yin *et al*. (2008) found that in order to classify a biosurfactant as a viable compound to treat hydrocarbon spills, it would need to be stable at a pH range of 6-10 and there should be negligible deterioration of surfactant activity at salinity levels of 2-10 g NaCl per litre. In other words, even if the yield of biosurfactant were to be affected the surface activity should not be sensitive to these two environmental factors.

In order to understand the underlying principles behind surfactant effects on membranes, Francisco *et al*. (2007) studied the effect of trehalose-based glycolipids on phosphatidylcholine membranes. Trehalose based lipids are primarily produced by the *Rhodococcus* species. The experiments indicate that the lipids segregate to form domains. The interactions were found to increase the interlamellar distance, d, which could be a result of increased hydrogen bonding caused by the segregation. This could be the reason behind the proposed theory that trehalose lipids are capable of permeabilizing phospholipid membranes. More work needs to be carried out in order to

understand the exact underlying principle behind the effect of biosurfactants on membranes.

More work needs to be done in this field, but considerable work has been done on the Rhamnolipid produced by Pseudomonas species which suggest that the emulsifier production is induced by molecular signals that are involved in quorum sensing (Peypoux *et al*., 1999). If we could understand the need of microorganisms to produce surfactants, it would enable us to design specific reaction conditions by which maximum surfactant production can be achieved on an industrial scale. Fermentation conditions need to be optimized to get maximum surfactant yield. There are many studies that have been carried out to elucidate the reason for surfactant production by bacteria. Some of them are outlined in the review by Ron & Rosenberg in 2001. The proposed driving factors to surfactant production are:

1. In the case of bacteria that grow in the presence of hydrocarbons, it is known that the interfacial area between the hydrocarbon and the aqueous phase acts as a rate-limiting factor. There are doubts however over the effect of surfactant production as the quantity of surfactants produced would create negligible emulsification effects on the bulk phase of the liquid. It is believed that the individual bacteria create a microenvironment in which they are capable of increasing surface area.
2. There are many instances where the oil degradation is not achieved as expected by oil resistant organisms. This is because the hydrocarbons are often biologically unavailable. The production of surfactants would result in increased mobility and solubility of the C_xH_y, which could potentially increase microbial degradation (Veenadig *et al*., 1999). One of the main objectives of the use of

surfactants in bioremediation is to be able to overcome the bioavailability problems since most of the contaminants in nature are hydrophobic in nature.

3. Surfactants have also been found to help in bacterial adhesion on to surfaces. When cells secrete extra cellular surfactants, they could form a film on the surface and help in cell attachment (Neu, 1996).

Apart from these it has also been proposed that certain biosurfactants have antimicrobial activity as in the case of surfactin, which is one of the most studied biosurfactant, produced by *B*. *subtilis*. Surfactants have also been used for the removal of heavy metals from the culture medium (Herman *et al*., 1995), suggesting that bacteria may produce them in order to acquire certain trace metals required for cell functioning.

Biosurfactant production by *Rhodococcus* genus is evidently growth related. This can be explained by the fact that the production of new biosurfactant increases as the growth rate of the organism increases (Haddadin *et al*., 2009). This study also found that the test bacteria used namely *R*. *Ruber* and *R*. *erythropolis* were able to cope with extreme pH ranges and could degrade almost all carbon sources.

1.3.2 Factors that Affect Biosurfactant Production:

There are a number of factors that affect the nature of the surfactant produced. Microorganisms are known to change the chemical composition of the surfactant depending on various factors such as:

1. In immobilized cell systems, the nature of the carrier or support material affects the rate of surfactant production. In general the carrier is responsible for the

water content present in the surfactants proximity. This affects the thermal stability of the surfactants. Hence the carrier should not retain any more of water than essential. Another problem encountered is, in the case of hydrophilic support material, they tend to restrict the hydrophobic substrate from reaching the surfactant sites due to their hydrophilic nature and thereby reduces the accessibility of the substrate to the active surfactant sites. In general hydrophobic materials such as celite and bonopore are the preferred choice of support material (Tyagi & Gautam, 2006).

2. The carbon source utilized also has an effect on the nature of biosurfactant. Carbon sources generally are carbohydrates, hydrophobic carbons or hydrocarbons. The exact nutrient requirement for each organism should be identified in order to achieve maximum surfactant production (Tani *et al*., 1997).
3. Another factor is the nature of the nitrogen source. The usually preferred nitrogen sources are urea and ammonium salts. Geurra-Santos *et al*. (1984) showed that a nitrogen limited growth could result in over production of surfactants as well as change in structure.
4. Other aspects of the fermentation conditions also affect the surfactant yield. Conditions such as pH, temperature, agitation and oxygen availability also play an important role. For example increase in agitation speed reduced the surfactant yield in *Nocardia erythropolis* whereas in yeast the surfactant yield increased with greater aeration rates and stirred speed (Gerson *et al*., 1979).

1.3.3 Advantages of Biosurfactants Over Existing Chemical Surfactants:

As discussed earlier surfactants are a group of industrially important chemicals. Petrochemical based surfactants are currently being employed in several fields such as food processing, domestic applications, oil clean-up and oil recovery, cosmetic applications and many of the allied industries. So why is there so much interest towards biologically derived surfactants? Currently there has been a lot of concern about the environmental impact of these chemicals. A study by Banat (1994) showed that the chemical surfactants that were tested were not biodegradable and hence could contaminate the environment. There are a vast range of chemicals such as pesticides, organic liquids (solvents from dry cleaning), oils (lubricating oils, automobile oils, hydraulic oils), heavy metals such as lead, cadmium, chromium, nickel generated due to waste leachates and sludges that find their way into the environment that consequently leads to a negative impact on the flora and fauna (Mulligan, 2005). Taking this into consideration, there are some very promising advantages in the use of surfactants of microbial origin. However the premise that biosurfactants are less toxic than their chemical counterparts is being contended right now due to insufficient data on this regard (Philp & Lang, 1998). In a survey conducted by Poremba *et al*. (1991) on eight synthetic and nine biosurfactants, the biosurfactants were found to be less toxic and more biodegradable, however more research needs to be conducted in this area. Some of the driving factors for biosurfactant use and production are: (Kosaric, 1992; Tyagi & Gautam, 2006).

1. Biodegradability: In general microbial surfactants are theorized to be easily biodegradable which makes them suitable for environmental applications such as bioremediation and oil clean-up.

2. Specificity: The ranges of biosurfactants that are available are as diverse as the range of organisms that are biosurfactant producers. Hence due to the chemical diversity of naturally produced amphiphile molecules this offers a wide selection of surface-active agents, which could be chosen to suit specific applications.
3. Biocompatibility and Digestibility: Since they are biologically derived they are theorized to be easily biodegradable and hence are the best choices for human related applications such as cosmetics, pharmaceutical production and in the food manufacturing and processing industries. This would be considered an important parameter, as this would ensure that the risk of any residue in the finished product would be reduced.
4. Availability of raw materials: One of the biggest advantages could be the ability of microorganisms to grow on a large number of substrates. This means that surfactants could be produced from cheap raw materials such as bagasse, whey, and waste oil, which are readily available in large quantities. The raw material could be chosen according to the application industry of the surfactant. For surfactants that are not required in great purity and for bulk applications like oil industries, we could choose low-end waste products as production media.

Given the fact that microorganisms are capable of growth in a wide variety of substrates, there is another possible approach for economical production of biosurfactants. According to Kosaric (1992), if we were to use organisms that could grow on organic matter available in industrial and municipal wastewaters, a double benefit would be obtained. In the process of effluent treatment, a valuable commercial product could be obtained. Although this cannot be considered as a large-scale surfactant production method, we could reduce the cost of effluent treatment by generating profit through the sale of the produced surfactant. In the case of anaerobic

processes there is another added advantage. When anaerobic microorganisms are used to produce biosurfactants from municipal effluent, the feedstock used is available throughout the year at very nominal prices and the energy requirement for the process can be partially met by the biogas that is generated.

In order for biosurfactants to compete with chemical surfactants on the market it must be economical to produce. The key to enhance biosurfactant production lies in media optimisation (Mutalik *et al*., 2008). In an experiment carried out using *Rhodococcus* sp. it was found that in the presence of multiple carbon sources, a relatively high level of hydrocarbon content is required for higher biosurfactant production. It was reported that the optimisation of the media resulted in the increase of biosurfactant yield from 3.2 g/L to 10.9 g/L representing a 3.4 fold increase in production. Statistical models were used to achieve this increased production through medium optimisation.

A lot of research still needs to be conducted to optimize the biosurfactant production and process optimisation in order to compete with the cheap chemical alternatives produced from petroleum derivatives that are available in the market.

1.3.4 Applications of Biosurfactants:

As discussed in the previous section there are many advantages of using biosurfactants. Surfactants have application in a varied range of industries. Some of the major applications and recent work carried out are discussed below.

1. Microbial de-emulsification of oil emulsions: One of the major problems facing the oil industry is the formation of oil-water emulsions during the various stages of oil recovery (Manning & Thomson, 1995). This is a major problem, as the emulsions have to be de-emulsified before the crude oil can be transported to refineries. At present, mixes of physical and chemical steps are used for de-emulsification but are very capital intensive (Manning & Thomson, 1995; Grace, 1992). There are many organisms that are capable of a high level of emulsifying activity (Singh *et al*., 2007) and in particular R. aurantius, Bacillus subtilis and micrococcus sp. can effectively de-emulsify petroleum mixtures (Kosaric, 1992). There are other oil processing operations of which particular interest is on microbial enhanced oil recovery (MEOR). MEOR is discussed in more detail in the last section of this chapter.

2. Effect of surfactant on bioremediation: As said previously one of the main problems of poor rate of biodegradation is due to contaminant aqueous insolubility or inaccessibility due to soil adsorption. Hence chemical surfactants have been used in a study to show an increase in the rate of biodegradation (Ward & Hamme, 1999). If we are able to produce relevant biosurfactants easily these applications can be extended to use biosurfactants instead of chemicals.

3. Degradation of PAH's: Polycyclic or polyaromatic hydrocarbons are produced as a side product of petroleum refining, coke production, and wood preservation (Park *et al*., 1990) and many of them are considered to be carcinogenic in nature. The addition of chemical surfactants or biosurfactants can increase the solubility of these chemicals. Vipulanandan & Ren (2000) showed that addition of a Rhamnolipid and sodium dodecyl sulphate (SDS) increased the solubility of Naphthalene 30 times. However it also increased the degradation time as compared to Triton X-100, due to the utilization of SDS as a carbon source by the degrading organisms. Another study by Deschenes *et al*. (1994) showed that another rhamnolipid isolated from a bio-slurry could improve the Naphthalene degradation in comparison to SDS but was only effective on PAH's with four aromatic rings or fewer. These studies show that there is always a possibility of discovering more effective surfactants, which could enhance biodegradation of these highly carcinogenic and stable pollutants.

4. Removal of heavy metals: According to Herman *et al*. (1995) rhamnolipids class surfactants could be used in the removal of heavy metals from contaminated soil due to their ability to form biosurfactant-metal complexes. The removal of heavy metals such as cadmium and lead in artificially contaminated laboratory soil samples were as high as 80%-100% whereas when conducted on actual field trials the results obtained were in the range of 20%-80% effective (Fraser, 2000). Another method of removal includes the ability of microorganisms to convert heavy metals to volatile forms through processes such as alkylation. As unlike traditional processes microorganisms cannot degrade heavy metals but their uptake is aided by the presence of surfactants. For instance anionic surfactants that come into contact with heavy metals lead to their desorption, cationic

surfactants reduce the association of metals by competing for the negatively charged surfaces. In all these cases the surfactant-metal union would need to be removed from the soil (Christofi and Ivshina, 2002). Mulligan (2005) conducted studies using a technique called micellar-enhanced ultrafiltration. This study looked at removing heavy metals from water samples using various concentrations of biosurfactant surfactin by 50,000 Da molecular weight cut-off ultrafiltration membrane. It was found that the metals (cadmium and zinc) associated with surfactin and formed micelles and remained in the retentate and did not pass through the membrane.

5. Biosurfactants could be used in the food industry as food additives: Locithin and derivatives, fatty acid esters, ethylene glycol are all currently being used throughout the world as food additives. A new emulsifier from Candida utilis has been successfully used for salad dressings (Roller *et al*., 1995). Biosurfactants are suitable candidates for this application since they are capable of serving as functional food additives. Biosurfactants also find applications in the healthcare and cosmetic industry (Brown, 1991). A whole range of compounds for cosmetic applications is being prepared by enzymatic conversions and whole cell transformations (Klibanov and Therisod, 1986; Banat & Desai, 1991). Those surfactants with saturated acyl groups are preferred over unsaturated ones as cosmetic industries require the compounds to have a minimum of three years of shelf life. Similarly surfactants have been proposed for use in the pulp and paper industry (Ron *et al*., 1989) as well as in the coal and textile industry (Griffin *et al*., 1990).

6. Biosurfactants used as an anti-adhesive: A recent published study has explored the possibility of using biosurfactants as an anti-adhesive to prevent growth of biofilms. Biofilms are a matter of concern to a host of industries such as food, medical and pharmaceutical since bacteria are able to colonize on most surfaces. Furthermore biofilms are not easily affected by sanitizing and antimicrobial agents when compared to free cells. The adhesion of bacteria onto surfaces is the first step towards biofilm formation (Donlan and Costeron, 2002).

   Zeraik and Nitschke (2010) found that when a surface was conditioned with surfactin (a well known biosurfactant) it became more hydrophilic and led to an expected decrease in microbial attachment. They further illustrated the advantages this could have in the field of biofouling. A decrease in temperature increased their efficiency, which could have implications in marine biofouling on ships. It was demonstrated that the application of surfactin at a temperature of around 4°C reduces the bacterial adhesion by 63-66%.

7. The robustness of the biosurfactants and their ability to remain active under extreme low temperatures allows some special applications. When treating oil-contaminated soils in Polar Regions, often a problem encountered is the increased viscosity and decreased water solubility making it difficult for indigenous bacteria. This could be solved by the use of emulsifiers or biosurfactants (Aislabie *et al*., 2006).

1.3.5 Biosurfactant Detection and Extraction Methods:

Due to the varied applications of biosurfactants it is necessary to have an easy, reliable and rapid method to detect the biosurfactant production by potential bacteria (Youssef *et al*., 2004). According to Youssef *et al*. (2004) the use of the properties of surfactants to measure surface activity instead of surface tension can lead to potential problems. Even though it is known that there is a direct relationship between the surface activity and emulsifying effect (Cooper & Goldenberg, 1987) it is not a certainty that surfactants capable of forming stable emulsions will lower the surface tension (Van dyke *et al*., 1993; Denger & Schink, 1995). There are a number of tests available to test the surface activity of surfactants and the more reliable methods are based on surface properties.

Examples of these methods are surface or interfacial tension measurements (Haba *et al*., 2000), axisymmetric drop shape analysis profile (van der Vegt *et al*., 1991), glass slide test (Persson & Molin, 1987), drop collapse method (Jain *et al*., 1991) and the oil spreading technique (Morikawa *et al*., 2000). The reason for the development of these methods is that, traditionally surface tension measurements were carried out but this was time consuming and hence proved to be a difficult task to perform for large number of potential bacterial isolates. Each of the other methods have distinct advantage in being simple and easy to carry out for the purpose of screening.

The drop collapse method developed by Jain *et al*. (1991) works on a principle that a drop of biosurfactant containing solution when placed on a thin layer of oil would collapse and spread evenly over the surface of the oil. This could be used to screen large number of samples. The oil spreading technique (Morikawa *et al*., 2000) works on the

principle that a drop of surfactant placed on a thin oil water surface would spread evenly and the extent of spread measured by the diameter of the circle formed is proportional to the strength of the surfactant. Youssef *et al*. (2004) used a large number of potential isolates to test the accuracy of three promising detection methods namely the oil spreading technique, drop collapse method and blood agar lysis. The results indicated that the oil-spread technique was very effective in biosurfactant detection and also found it much easier to standardize and use than surface tension measurements. The drop collapse method was found to be reliable in detecting a slightly higher concentration of surfactant but was not effective in detecting surfactants of very low concentrations. The blood agar lysis method tested negative with many of the strains that were tested positive for the other two tests. Hence it is possible that the other two methods oil spread plate technique and drop collapse method could be used for general screening purposes and the surface tension could then be measured for confirmation.

One of the major drawback with the commercial production of biosurfactants is the recovery steps needed to obtain purified product, which is true for almost all the biotechnological processes. Considering this, bioprocess development is one of the primary objectives for the commercialization of biosurfactant production. Bioprocess optimisation of a process involves process optimisation, raw material selection and identification of fast, efficient and cheap product recovery methods. According to Sen *et al*. (2006) in industries such as pharmaceuticals 60% of the total costs of production are invested on downstream processing such as membrane ultrafiltration, ion exchange chromatography, adsorption on polystyrene resins and other similar expensive processes. There are wide ranges of biosurfactant recovery processes that have been widely reported such as solvent extraction, acid precipitation, crystallization, centrifugation and ammonium sulphate precipitation, which would be considerably

cheaper alternatives (Desai and Banat, 1997). Some of the commonly used and researched extraction methods are presented in the table below:

Downstream recovery procedure	**Biosurfactant property responsible for separation**	**Instrument/apparatus/ setup required**	**Advantages**
Acid precipitation	Biosurfactants become insoluble at low pH values	No set-up required	Low cost, efficient in crude biosurfactant recovery
Organic solvent extraction	Biosurfactants are soluble in organic solvents due to the presence of hydrophobic end	No set-up required	Efficient in crude biosurfactant recovery and partial purification, reusable nature
Ammonium sulfate precipitation	Salting-out of the polymeric or protein rich biosurfactant	No set-up required	Effective in isolation of certain type of polymeric biosurfactants
Centrifugation	Insoluble biosurfactants get precipitated because of centrifugal force	Centrifuge required	Reusable, effective in crude biosurfactant recovery
Foam fractionation	Biosurfactants, due to surface activity, form and partition into foam	Specially designed bioreactors that facilitate foam recovery during fermentation	Useful in continuous recovery procedures, high purity of product
Membrane ultrafiltration	Biosurfactants form micelles above their critical micelle concentration (CMC), which are trapped by polymeric	Ultrafiltration units with porous polymer membrane	Fast, one-step recovery, high level of purity
Adsorption on polystyrene resins	Biosurfactants are adsorbed on polymer resins and subsequently desorbed with organic solvents	Polystyrene resin packed in glass columns	Fast, one-step recovery, high level of purity, reusability
Adsorption on wood-activated carbon	Biosurfactants are adsorbed on activated carbon and can be desorbed using organic solvent	No setup required, can be added to culture broth, can also be packed in glass columns	Highly pure biosurfactants, cheaper, reusability, recovery from continuous culture
Ion-exchange chromatography	Charged biosurfactants are attached to ion-exchange resins and can be eluted with proper buffer	Ion-exchange resins packed in columns	High purity, reusability, fast recovery
Solvent extraction (using Methyl tertiary-butyl ether)	Biosurfactants dissolve in organic solvents owing to the hydrophobic ends in the molecule	No set-up required	Less toxic than conventional solvents, reusable, cheap

Table 1.1: Physiochemical property based biosurfactant recovery methods and their relative advantages (Sen *et al*., 2006).

Other separation methods include ultrafiltration, foam fractionation, ion-exchange chromatography and adsorption-desorption on wood based activated charcoal and on polystyrene resins. One of the main advantages of these is that they are suitable for continuous operation and yield a high degree of purity (Sen *et al*., 2006). Most of the solvents that were used in surfactant extraction previously for example acetone, methanol, and chloroform are toxic in nature and contribute towards environmental pollution. Cheap and less toxic solvents such as methyl tertiary-butyl ether (MTBE) have been successfully used recently to remove surfactants produced by *Rhodococcus*. A study by Kuyukina *et al*. (2001) compared the various solvent extraction systems. By comparing the extraction efficiency of MTBE, chloroform-methanol and dichloromethane it was found that MTBE efficiency was much higher (80%) as compared to chloroform-methanol (72%) and dichloromethane (44%). These kind of low cost, less toxic and more readily available solvents can be used to reduce the environmental impact and expenses substantially.

In many cases it is often required to use more than one extraction process. Multiple downstream processing steps might be required. Multi-step recovery strategies may prove more effective (Reiling *et al*., 1986). By using such a multi-step process, it would be possible to recover biosurfactants of a certain level purity that is required for our purpose. For instance the crude products at the initial stages of recovery could be used in environmental applications and oil recovery operations where a high degree of purity is not required, whereas the further stage extracts could be used in pharmaceutical and food industries where a high degree of purity is absolutely necessary. This is crucial as the increased number of steps required to purify the surfactant would increase the cost of production.

With the advent of advanced genetic engineering and molecular biology tools we should be able to produce recombinant species that are much more capable of producing higher yields of biosurfactants (Sen *et al*., 2001).

1.4 Use of Mixed Culture Bacterial Systems:

The limitations of the use of single cell biotransformations led to research, involving the use of a consortium of bacteria, which were selected to exploit the individual properties of each strain. Manoj *et al*. (2006) proposed the use of two strains of bacteria, one that forms an excellent emulsion (*Pseudomonas aeruginosa* DHT-DL isolated from soil sample) and another strain (*Pseudomonas putida* 5a1) that efficiently degrades pure hydrocarbons. Through this method they were able to obtain a bacterial consortium with a combination of superior emulsification and degradative capabilities. Other researchers have carried out work in this area with many cases of superior degradative capabilities coming from combinations rather than the individual strains themselves (Suguira *et al*., 1997; Bicca *et al*., 1999; Rahman *et al*., 2001; Rahman *et al*., 2002; Solanas *et al*., 2004). Hamme & Ward (1998) tested the ability of a consortium of bacteria to degrade crude oil in the presence of a chemical surfactant. Almost all of this research was carried out in order to ascertain whether the use of a consortium of bacteria individually selected on their ability to excel at a particular form of degradation would enhance the rate of degradation of the pollutants. Much of this work has been carried out on variety of bacterial strain but the research presented here is based on the use of two strains of bacteria of the *Rhodococcus* species, namely *Rhodococcus ruber* and *Rhodococcus opacus*.

The focus on the use of a consortium of bacteria has been researched mainly using organisms of the *Pseudomonas* genus (Rahman *et al*., 2001; Rahman *et al*., 2002; Abouseoud *et al*., 2008) and Cornybacterium species (Rahman *et al*., 2002). Another aspect that is to be considered is that, work carried out in the *Rhodococcus* genus has been mainly focused on the use of rhamnolipid surfactants. These are predominantly the kind of surfactants produced by *Rhodococcus erythropolis* (Rahman *et al*., 2002, Rahman *et al*., 2003; Pirog *et al*., 2006; Peng *et al*., 2007). There has been a lesser quantity of research work directed towards understanding the biosurfactant capabilities of the *Rhodococcus ruber* strain (Bicca *et al*., 1999; Philp *et al*., 2002). Christofi *et al*. (1998) carried out research using both the strains of bacteria that have been investigated in this study, namely *Rhodococcus opacus* and *Rhodococcus ruber*. In this study they were able to analyse ten strains of bacteria belonging to the *Rhodococcus* genus and identify the nature of the various surfactants produced on a hydrocarbon substrate.

In the use of mixed microbial cultures there are many methods of hydrocarbon uptake including uptake of water-soluble hydrocarbons, biosurfactant-mediated transfer and direct adherence to hydrocarbon droplets. Consequently this makes understanding the effect of addition of chemical or biological surfactant to the culture media difficult, since each microbial population reacts differently. A clear understanding of these behaviour patterns are required in order to use mixed culture systems effectively (Van Hamme and Ward, 2001).

Van Hamme and Ward (2001) were able to prove that each organism responds differently to addition of biosurfactant depending on the individual circumstances. For instance the addition of a chemical surfactant increased the hydrocarbon uptake of a Pseudomonas spp. This was because it increased the bioavailability of insoluble

hydrocarbons. On the other hand the addition of chemical surfactant into the culture medium of *Rhodococcus* sp. resulted in reduced rate of degradation. This was because it caused a disruption to the cell-oil interaction, which is the preferred hydrocarbon uptake route for this particular microbe whereas the previous strain was easily able to form emulsions in liquid medium. The chemical surfactant inhibited the attachment of the bacteria on to the oil-water interface. Hence careful study of the individual organisms is required in order to evaluate the effectiveness of a mixed culture system.

Although the addition of biosurfactant increases the solubility of certain water insoluble hydrocarbons, there are conflicting reports of its advantages (Dean *et al*., 2001). In an experiment carried out by Dean *et al*. (2001) it was demonstrated that the presence of a surfactant in fact reduced the degrading effect of phenanthrene on one strain although it increased the phenanthrene bioavailability. When grown as a co-culture with surfactant additive, the overall efficiency was increased but this was due to preferential advantage obtained by the other strain. These experiments further stress the importance of testing mixed culture interactions. Due to diverse range of combinations that are possible, mixed culture systems are selective and have high degree of specificity. This is because each organism that has been selected to form a mixed culture system can be chosen for a very specific ability and hence can be made very selective in the overall capability of the mixed culture bacterial system.

In spite of the many pitfalls, the use of bacterial consortium which are not antagonistic to each other would invariably result in higher rates of substrate degradation. When two strains of *Rhodococcus* genus, namely *Rhodococcus ruber* and *Rhodococcus erythropolis* were used in a mixed culture experiment to degrade diesel oil, the rate of degradation of the diesel was superior when the organisms were

cultivated together as compared to experiments where they were cultivated as pure cultures. Where *R. erythropolis* was easily able to form suspensions, *R. ruber* was found to cling onto oil-water interface. This difference in uptake mechanism paved the way for enhanced degradation than when individually grown (Zhukov *et al*., 2006).

The major limitation in the degradation of hydrophobic components is their low water solubility thus making them unavailable to the microorganisms. This was evident in the study carried out by Abalos *et al*. (2004). A bacterial consortium was used to degrade model casablaca crude oil with and without the presence of rhamnolipids produced by *Pseudomonas aeruginosa*. While there was hardly any difference in degradation of easily degradable substances such as n-alkanes, considerable difference was found in the degradation of complex mixtures such as pristane and phytane. Rhamnolipid increased the rate of degradation from only 16.25% in 20 days to 70% thus proving the main obstacle to degradation is the low solubility. The same was the case for other components such as naphthalene and PAH along with methyl and dimethyl derivatives.

1.5 Microbial Enhanced Oil Recovery:

In traditional oil production sites when the cost of production and recovery of one barrel of oil exceeds the price of crude oil in the market, the oil well is abandoned, often with around 70% of the oil still remaining. It is a widely accepted fact that only around 30% of the oil present in the reservoir is recoverable using traditional oil recovery processes (Singer & Finnerty, 1984). According to the current figures the quantity of oil unutilized due to this is about 377 billion barrels with another 37 billion barrels that

cannot be recovered by traditional methods (Cano Petroleum Inc., 2008). There are many factors that goes into consideration when an oil well is abandoned and poor oil recovery could depend on several factors.

Since most of the oil in the wells are non-recoverable and due to the increasing price of crude oil in the market, alternative methods of Enhanced Oil Recovery (EOR) were devised (Morkes, 1993). Some the newer methods of EOR include chemical treatment (as mentioned before), gas flooding, thermal processes, water flooding, reverse wettability, seismic wave stimulation and microbial methods.

Biosurfactants are amphillic molecules that contain both the hydrophilic and hydrophobic components in their structure, thus allowing them to partition at the interface of two fluids with different polarities. This could be a water-oil or water-air interface thus making them ideal candidates for enhanced oil recovery.

Microbial Enhanced Oil Recovery (MEOR) operates under the principle that microbes ferment the hydrocarbons to produce a side product that helps in the recovery of crude oil by mobilizing and detaching the oil from the porous rock material. The applications of MEOR are varied and are not limited to oil recovery alone. Other applications include oil well work-over, the stimulation of reservoirs and oil wells, replacing more expensive conventional methods as well as earth surface application for oil spills and petroleum hazardous site clean-up (Cano Petroleum Inc., 2008).

MEOR can be operated in the following three ways:

1. The first could involve the direct injection of the microorganism into the reservoir through the wells (Bubela, 1985).
2. Another method is to inject economically cost effective nutrients into the reservoir. This helps in stimulating the naturally growing biosurfactant producing organisms.
3. The third method is to produce surfactants ex-situ by fermentation in a reactor and then injecting the purified product into the reservoir.

The use of microbial-based solutions is also advantageous due to the following reasons:

1. The by-products are environmentally friendly and non polluting.
2. In the case of utilizing already existing bacterial populations, only low cost nutrients need to be supplied in order to stimulate them and allow them to grow in great numbers.
3. Although frequent monitoring is required, the process once initiated is self-sustaining and can be utilized for prolonged periods without much intervention.

A lot of work has been initiated by interest groups due to financial rewards of undergoing such a project and the benefits it would have on the practical field applications. Although as of now there are no regulations in place for the implications of MEOR on test fields, it should be carried out with the local federal regulations. Douglas *et al* (1988) concluded after extensive studies that MEOR is safe to conduct as long as sufficient steps are taken to control the spread of possible pathogenic indigenous organisms and monitoring devices are installed. The use of MEOR in place of chemical surfactants is now being seriously considered due to the hazardous nature of chemical

surfactants and the effects that the residues of chemical surfactants are likely to have on the environment.

Considerable amount research has been carried out at laboratory scale in order to study the effect of surfactants and the potential application in the field of MEOR. Typically experiments would be carried out utilizing core samples and columns containing the substrates that are of interest, which would usually be soil samples from potential areas where MEOR could be applied. In an investigation carried out by Chang (1987), *C. acetobutylicum* was injected into core samples, which were contaminated with oil that was added. Results showed that there was 20% increase in the oil recovery when compared to washing the column with a simple nutrient solution. Similar work was carried out by Adkins *et al*. (1992) using two strains of bacteria namely *Vibrio aspartigenicus* and *Bacillus licheniformis*. Results showed that when using *V. aspartigenicus* to recover residual oil from Viola limestone cores (20 – 50 mesh) a 32-36% increase in oil recovery was observed as compared to control, which had undergone a three treatment process (nutrient injection, incubation and flooding with brine). Similarly when the same experiment performed using *B. lichniformis* showed an increase in oil recovery by 27% when compared to the control. In a recent study Bordoloi and Konwar (2008) used biosurfactants produced by *P. aeruginosa* to observe the release of oil from a column of dry sand that was saturated with crude oil. Results showed that at the three temperature ranges that were tested (room temperature, 70°C, 90°C) there was an increase of 49-62% increase in the oil recovery when compared to control which was treated with just the culture medium.

Similar work has also been carried out in the field to study the efficiency of MEOR in real world applications. An early work carried out on unconsolidated water flooded

sandstone reservoir in USA provided very conclusive results. Molasses was injected into the reservoir that included an inoculum of *Clostridium acetobutylicum*. Results showed that after the injection the oil production increased by 250% (Tanner *et al*., 1991). A recent study carried out by Jinfeng et al. (2005) in the Dagang oil fields in China investigated the use of three organisms namely *Arthrobacter spp*, *Pseudomonas spp*. and *Bacillus spp*. A bacterial suspension of these three organisms along with a nutrient solution was pumped into the reservoir and the oil output measured. After an initial drop of 21% in the oil recovery from 55 t/day to 30 t/day the oil recovery significantly increased in all the 7 oil wells that were tested and produced an additional 8700 t of oil over a six-month period.

In order to use MEOR a lot of work still needs to be carried and for MEOR to be accepted widely, we would need to address some of the concerns that surround the use of MEOR. Some of them are:

1. The injection of nutrients into the reservoir should in no way aid the growth of indigenous organisms and the proliferation of pathogens. Also any injected bacteria should be screened for harmful consequences and should be completely non-pathogenic in nature.
2. There should be no leakage of the injected microorganisms into the underlying aquifers.
3. The water effluent that is generated during the MEOR process presents a problem, as it is rich in organic material, which is mostly biomass. This cannot be directly disposed as this would lead to contamination of the ground water source and also could lead to eutrophication.

(Sharma & Georgiou, 1993)

To conventionalize the use of MEOR and maximise the future prospects research needs to be carried out to address some of the issues such as:

1. Better understanding of the mechanisms of oil recovery. It is not easy to scale up using data from laboratory scale experiments. It is likely that different results would be obtained using different cultures from the same reservoir.
2. The possibility that bacterial could plug the reservoirs is a major concern to oil operators where every effort is taken to minimize fouling. More research needs to be conducted in order to convince that in sufficiently low concentrations, the metabolites produced would help in increasing oil recovery. This problem is further compounded by the fact that the injection of nutrients could stimulate the growth of undesired indigenous microorganisms. This could have an adverse effect on the oil recovery and could lead to formation of unwanted products such as Hydrogen Sulphide.
3. More mathematical models need to be constructed from laboratory and field trials in order to evaluate the efficiency of MEOR on new sites. This should take into consideration the microbial growth kinetics, microbial transport phenomena and metabolism rates. In spite of research being carried on for several years, it has not been possible to produce a completely satisfactory scale up model.

(I.M. Banat, 1994)

Once these hurdles have been overcome there is a tremendous potential for MEOR in the oil industry.

1.6 Aims:

Broadly the aims and objectives of this body of work can be summarised as follows:

1. Chapter 2 details the work carried out in the initial stages of project such as media preparation and general maintenance of the bacterial cultures. The techniques used in bacterial quantification have been detailed. The aim of this chapter is to carry out bacterial adaptation in solvents namely methanol, glycerol, n-hexadecane and assess the impact of various glycerol concentrations on growth.
2. Chapter 3 deals with three aspects of the optimisation process, which are optimum temperature, nitrogen source and pH range. This chapter also details the effect of various NaCl concentrations on the growth rate. The aim of these experiments is to establish the optimum temperature, nitrogen source and pH range that would elicit the maximum surfactant production from the bacterial strains.
3. Chapter 4 details the work carried out in order to characterise the nature of the surfactant being produced by the two bacterial strains *Rhodococcus opacus* and *Rhodococcus ruber*. The aim of this chapter is to characterise the surfactant being produced and determine if the surfactants would be beneficial in aiding hydrocarbon uptake.
4. Chapter 5 discusses the operation of mixed culture bacterial systems. The aim of this chapter is to assess the efficiency of mixed culture bacterial systems that make use of conventional mixed culture reactor and membrane reactors where the different bacterial strains are physically separated from each other.

In this study, we investigate the possibility of using *Rhodococcus ruber* and *Rhodococcus opacus* to produce surfactants in presence of hexadecane as the sole carbon source. Prior to the use of hexadecane as the sole carbon source, the solvent tolerances of the bacterial strains to solvents such as methanol and glycerol will be tested. Recent research has demonstrated that the growth conditions and medium composition have a great impact on the quantity and nature of surfactant that is being produced (Moussa *et al*., 2006; Abouseoud *et al*., 2008). It has been shown that the nature of the nitrogen and carbon source, along with the growth parameters such as shaking speed and incubation time significantly alter the quantity of the surfactant that is being produced. This study has undertaken various medium optimisation experiments in order to ascertain the best-suited nitrogen source and shaking speed, which would elicit the maximum quantity of surfactant release from the bacteria. Alkanes are considered the preferred source of carbon for the production of surfactants. "A common physiological response in alkanotrophic bacteria is to elicit biosurfactant molecules when presented with n-alkanes as sole source of carbon" (Philp *et al*., 2002).

This study investigates the ability of *Rhodococcus ruber* and *Rhodococcus opacus* to use four main sources of nitrogen (namely ammonium sulphate, ammonium nitrate, sodium nitrate and potassium nitrate). The quantification of the surfactants has been carried out using the Phenol-Sulphuric acid assay that has been described by Taylor (1995). This study also looks into the aspect of the drop in pH, which was observed in the ammonium salts. In order to elucidate the effectiveness of the surfactants that are being produced, a range of tests have been utilized. These include the emulsification ability, the degree of adhesion to hydrocarbons and surface tension measurements. The various surfactant extractions methods are also discussed in detail, among which the solvent extraction method was chosen to be undertaken in this project due to relative

ease of experimental setup and degree of purity that was required for the purpose of this project.

The final part of this project investigates the use of mixed culture bacterial systems in various experimental setups. In one method the two bacterial strains namely, *Rhodococcus opacus* and *Rhodococcus ruber* have been grown together in a conventional 3L automated batch reactor. The other method involved the use of the two strains in a specially constructed membrane reactor, in which by the use of membrane partitions the two bacterial strains share a common culture media but are physically separated from each other. To assess the behaviour of the two bacterial strains, prior to conducting the experiments in the 3L automated batch reactor, flask cultures were cultivated in varying concentrations of each strain.

Chapter 2: Growth Studies and Media Adaptation

This chapter describes the work carried out in the initial stages of the project such as media preparation and general maintenance of the bacterial cultures. The chemical compositions of the various media used for the bacterial cultivation and the techniques used to quantify the growth have been explained in detail. Bacterial adaptation in solvents namely methanol, glycerol and n-hexadecane is another aspect that has been discussed along with growth measurements carried out using a HPLC system.

2.1 Materials and Methods:

2.1.1 Chemicals:

The bacteriological agar was purchased from Difco (UK) and unless stated all the other chemicals were of laboratory grade and purchased from either Sigma Chemical Company (UK) or Fisher (UK).

2.1.2 Bacteria:

The bacteria used in these experiments were as follows: *Dechloromonas denitrificans* (DSM15892), *Rhodococcus opacus* (DSM 43250) and *Rhodococcus ruber* (DSM7511), which were purchased from DSMZ the German National Resource Centre for Biological Material and stored at 4°C prior to use.

Dechloromonas denitrificans (DSM15892) were grown on medium 830 which consisted of the following components per litre of deionised water; 0.5g of yeast extract, 0.5g of peptone (both supplied by Merck (UK)), 0.5g of Casamino acids, 0.5g of

glucose, 0.5g of corn starch, 0.3g of sodium pyruvate, 0.3g of K_2HPO_4 and 0.05g of $MgSO_4.7H_2O$. The pH of the medium was adjusted to 7.2 using KH_2PO_4.

Rhodococcus opacus (DSM 43250) was grown on medium 65 (Gym Streptomyces medium) which consisted of the following components per litre of deionised water; 4.0g of Glucose, 4.0g of yeast extract and 10.0g of malt extract (supplied by Merck (UK)).

Rhodococcus ruber (DSM7511) was grown on medium 220 (Caso agar) which consisted of the following components per litre of deionised water; 15.0g of peptone from casein, 5.0g of peptone from soymeal and 5.0 g of NaCl. The pH of the medium was adjusted to 7.3 using KH_2PO_4. This medium is identical to Tryptone Soya agar.

Medium B contains the following constituents per litre of deionised water at pH 7; 0.8g of K_2HPO_4, 0.2g of KH_2PO_4, 0.05g of $CaSO_4.2H_2O$, 0.5g of $MgSO_4.7H_2O$, 0.01g of $FeSO_4.7H_2O$ and 1g of $(NH_4)_2SO_4$. In addition the medium was supplemented with 10ml of SL-6 and the appropriate carbon source. SL-6 contained the following constituents per litre of deionised water 0.1g of $ZnSO_4.7H_2O$, 0.03g of $MnCl_2.4H_2O$, 0.3g of H_3BO_3, 0.2g of $CoCl_2.6H_2O$, 0.01g of $CuCl_2.2H_2O$, 0.02g $NiCl_2.6H_2O$, 0.03g of $Na_2MoO_4.2H_2O$.

Minimal Salts Medium (MSM) contains the following components per litre of deionised water at pH 7; 3.0g of $NaHCO_3$, 1.0g of NH_4HCO_3, 0.2g of K_2HPO_4, 102.5mg of $MgSO_4.7H_2O$, 36.75mg of $CaCl_2.2H_2O$, 10mg of $FeSO_4$ and 1ml of trace element solution which contains the following components per litre of deionised water; 100mg of $NiSO_4.H_2O$, 100mg $MgCl_2.4H_2O$, 100mg of $ZnSO_4.H_2O$, 100mg of H_3BO_3, 50 µg of

$CoCl_2.6H_2O$, 40 µg of $H_3PO_4.12MoO_3.24H_2O$, 10 µg of $CuSO_4.5H_2O$, 10 µg of $AlCl_3.6H_2O$ and 0.5 µg of EDTA.

In order to prepare petri plates, agar was added at 20g per litre for all the above media. Autoclaving was carried out at 121°C for 15 minutes except when the carbon source used was glucose in which case the autoclaving was carried out at 110°C for 10 minutes to prevent any breakdown of glucose.

2.1.3 Bacterial Enrichment and Maintenance:

The three strains of bacteria used in this project were inoculated aseptically into their respective recommended media as follows. The bacteria were supplied as lyophilised solids in sealed glass vials with the exception of *Dechloromonas denitrificans* (DSM15892) which was supplied on agar. For the lyophilised bacteria, the seal was broken using a glass cutter and 1ml of sterile deionised water was added. The water that has been used in the cultivation of the bacteria throughout this work was obtained from a Millipore Direct-Q5 system. Subsequently, 1ml of the recommended media was added and mixed thoroughly. Once mixed, 1ml was aseptically transferred to the recommended liquid media (100ml media in 250ml Erlenmeyer flasks) and the remainder of the liquid from the vials were used to create streak plates in the same media. *D. Denitrificans* was transferred to the liquid media using a sterile microbiological loop. All the bacterial cultures were initially grown at 20°C on a Stuart Orbital shaker (Stuart, UK) at 150rpm. The above liquid cultures were subsequently sub-cultured for six generations before any studies were carried out on them. On a regular basis streak plates of all the pure cultures were made and stored at 4°C in order to safe guard from potential contamination.

2.2 Bacterial Growth Measurements:

All the cell growth measurements were carried out spectrophotometrically. This was carried out by measuring the light absorbance in 1 cm light path in a plastic cuvette (Fisher Scientific, UK) at a wavelength of 600nm (OD600) using a Lightwave spectrophotometer (Lightwave, UK). The corresponding cell number was calculated using a haemocytometer slide 0.1 mm x 0.0025 mm (Superior, Germany) and an Olympus CH-2 or Olympus LH50A microscope was used with the latter being fixed with a JVC colour video camera which enables easier viewing of the bacteria.

An optical density reading at 600nm was taken before each count. A cover slip is slid over the microbial suspension and by working the cover slip to and fro on the slide until "Newton's rings" appear which ensures that the correct chamber depth is achieved. The microbial suspension is diluted if necessary and killed by using formaldehyde or 70% methanol if the cells are motile. To obtain statistically accurate data at least 200 or more bacterial cells should be counted. On the other hand if there are more than 20-30 cells per 50μm square, then a further dilution is carried out preferably around 10 times. The total average of the cells counted should be multiplied by 4 x 10^6 to get the number of bacteria per millilitre of the bacterial suspension. Any further dilutions carried out should be factored into the calculation.

The correlation between the OD600 values and the cell count was established for *R. opacus* and *R. Ruber* using haemocytometer counts as presented in Table 2.1 below. The optical density values were recorded and the corresponding cell counts were performed.

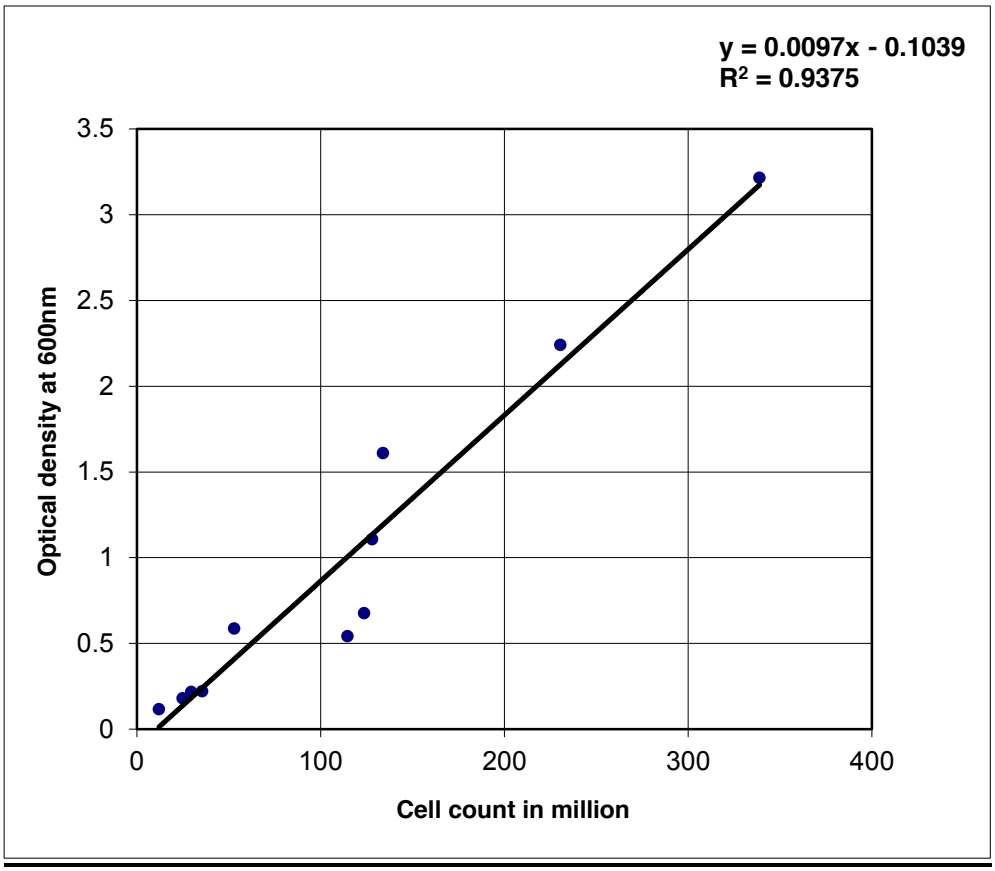


Figure 2.1: Optical density Vs. Cell Count

Organism	**Number of observations**	**Pearson correlation "R"**	**Significance "F"**
Rhodococcus opacus	11	0.937	<0.000001
Rhodococcus ruber	8	0.945	<0.000001

Table 2.1: Optical density Vs. Cell Count correlation

2.3 Microbial Adaptation in Solvents:

Almost all of the adaptation growth was carried out in 250mL Erlenmeyer flasks that were kept at 20C and shaken at 130 RPM in a shaker. All the strains of bacteria were grown on Medium B with only the carbon source modified in each case. For the glycerol adaptation to *R. opacus*, cells growing on Glucose as sole carbon source were split into liquid medium containing 2g/L of glucose and 1% (v/v) of glycerol. The cells were only split into the next generation after reaching the exponential phase. The amount of glucose was gradually reduced by 0.5g/L in successive generations with the 5^{th} generation growing only on glycerol as the sole carbon source. After this six generations of cells were cultivated before any growth studies were carried out. The 7^{th} generation of *R. opacus* growing on glycerol as the sole carbon source was taken for the growth studies at various percentages of glycerol.

The *R. opacus* in glycerol as sole carbon source was used to inoculate a medium containing small amounts of glycerol in its crude form, which was derived as by-product of biodiesel production. The crude glycerol was provided by Neil Hollow as part of another PhD project. This was also carried out until six generations of cell cultures were reached for further experiments.

R. opacus was grown on 2g/L of Glucose and 1% (v/v) of concentrated methanol. For each successive generation the amount of Glucose was reduced by 0.5g/L. On reaching the 5^{th} generation the amount of Glucose was maintained at 0.5g/L and the methanol concentration was increased by 0.5% (v/v) until 3% (v/v) of methanol was reached. After this the *R. opacus* was observed to give good growth on methanol as sole carbon source and further generations were produced.

The solvent adaptation growth of *R. opacus* in methanol was carried out in 250ml Erlenmeyer flasks which were kept at 25 °C and shaken at 150 RPM in a shaker as opposed to 20 °C and 130 RPM as carried out in the other solvent tolerance experiments. *R. opacus* was grown on Medium B with methanol as the sole carbon source. For the growth studies, the culture that was chosen for inoculation was the 30th generation of bacterial culture growing in slightly lower concentrations of methanol. In order to ascertain that there is no contamination, the generation prior to the source of inoculum was plated on to solid agar medium containing methanol which was added aseptically using sterile filter since methanol is not compatible with sterilisation at 121 °C. After visual verification of the growth colonies that were formed on solid media, further experiments were carried out.

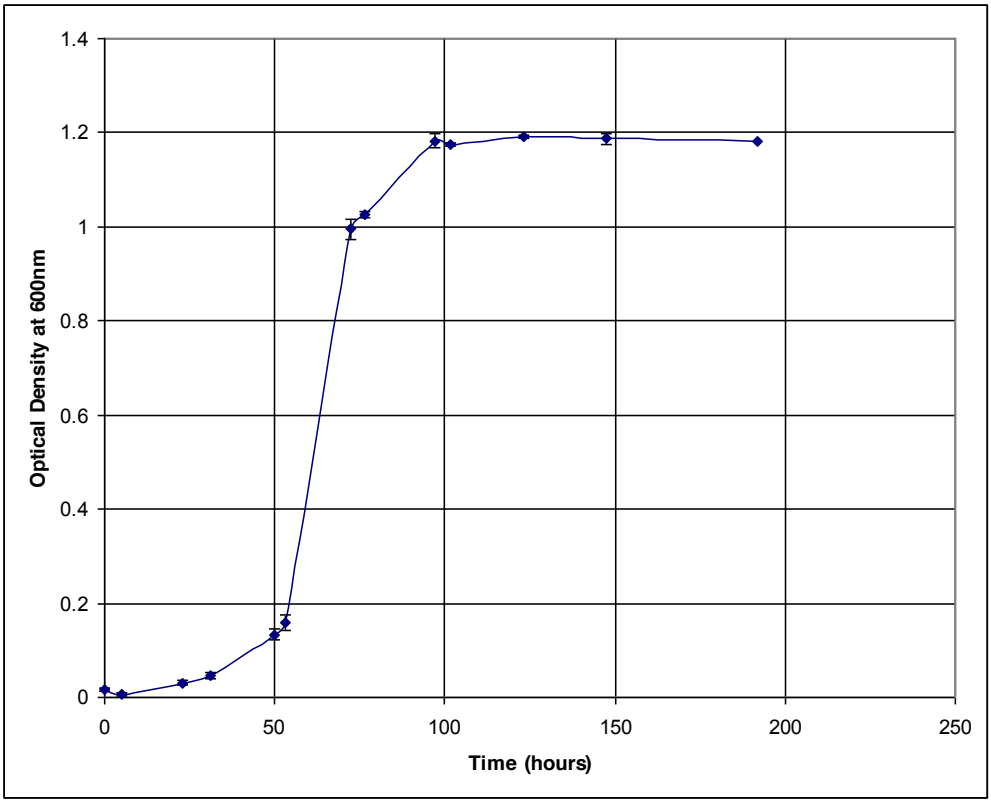


Figure 2.2: The growth obtained following the growth on methanol as the sole carbon source.

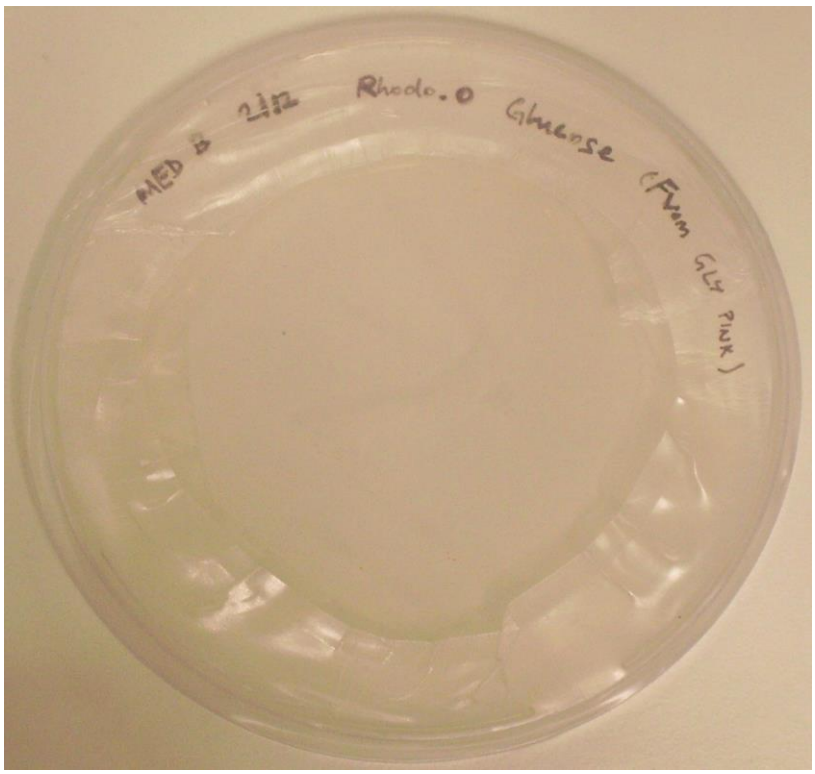


Figure 2.3: *Rhodococcus opacus* grown on glucose shows no change in colour

Figure 2.4: *Rhodococcus opacus* grown on glycerol produced pink strains

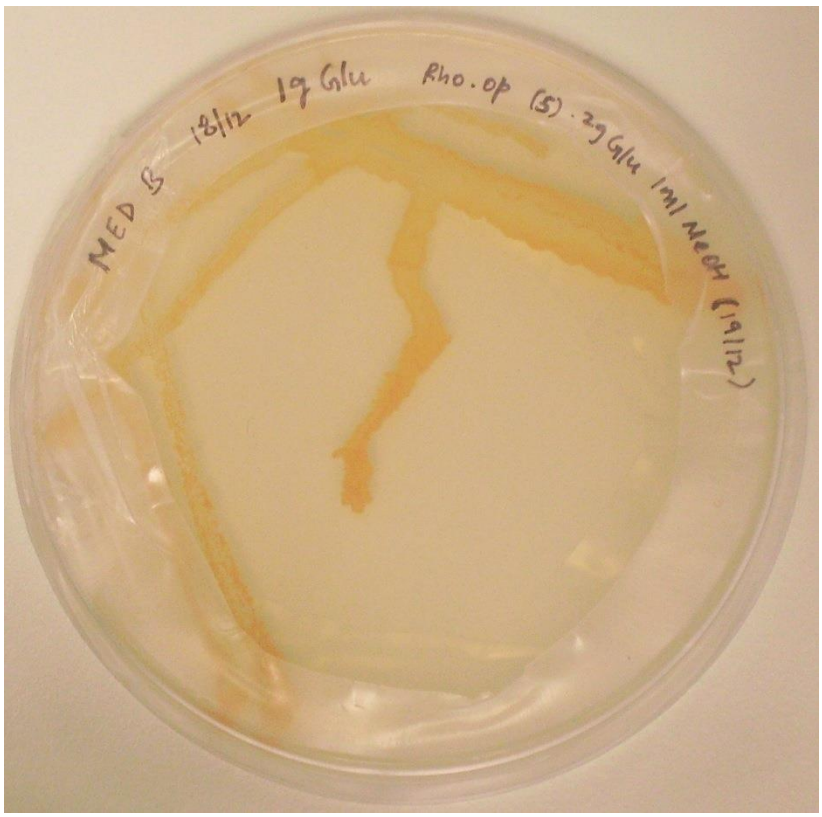


Figure 2.5: *Rhodococcus opacus* grown on methanol produced yellow-orange strains

During the growth of *Rhodococcus opacus* it was found that the bacteria produced different coloured strains, which was found to vary depending on the carbon source as illustrated in Figure 2.3, Figure 2.4 and Figure 2.5. This was observed mainly in the solid plate cultures but the liquid culture of *Rhodococcus opacus* produced a pink colour in all the carbon sources it was grown on (glycerol, crude glycerol, and methanol) but not in n-hexadecane. The reason behind this behaviour is still unclear. As discussed in Chapter 1 there could be a number of factors behind this behaviour such as the nature of the carbon source, medium composition, etc. The above observation could be due to the difference in the uptake process, which could be a different metabolic pathway due to the artificial unavailability of n-hexadecane leading to this colour change.

To study the hydrocarbon degradation of both the strains namely *R*. *opacus* and *R*. *ruber*, both the strains were grown on 1% (v/v) of n-hexadecane. *R*. *opacus* inoculum was taken from the well-adapted glycerol cultures to ensure that the bacteria are relatively solvent tolerant. *R*. *ruber* was taken from cells growing on glucose. To ensure that there is no contamination, samples were taken after the first generation of both the strains grown in n-hexadecane and were plated on to solid agar medium containing n-hexadecane. These samples showed well-developed single colonies without any sign of contamination. The inoculums for the second-generation liquid cultures were taken aseptically from these solid agar plates for each of the strains.

The adaptation of *Rhodococcus opacus* and *Rhodococcus ruber* in n-hexadecane was carried out for six generations in the presence of 1ml of the hydrophobic substrate. From the initial studies and observations it was noticed that the cells of both the species was found to grow only on the interface of the two immiscible phases. There was a striking partition of the cells in the liquid cultures. This was found to match with the literature work carried out on similar species of the same genus. According to Neu (1996) studies have shown that there is a striking partition of *Rhodococcus* cells into the oil phase when they are cultivated on an aqueous mineral media and a liquid hydrocarbon as the carbon source. Another interesting aspect that was noticed in these experiments was that large clumps were formed by *R*. *ruber*, whereas none were found with the *R*. *opacus* strain (refer to Figure 2.5 below). This is clearly visible in the liquid media culturing. Current literature review states that the presence of other surfactant molecules in the culture medium could decrease the rate of clumping

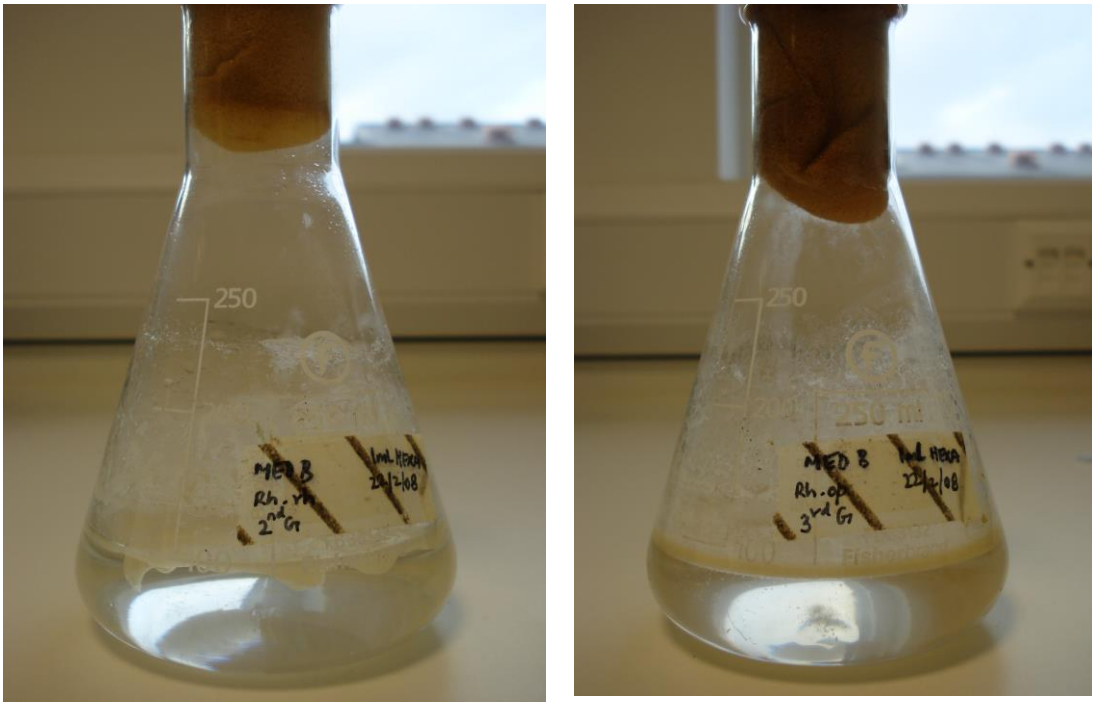


Figure 2.5: R. ruber and R. opacus cultivated in n-hexadecane

2.4 Growth Studies in Glycerol:

Whilst carrying out glycerol growth studies, it was found that the external morphology of the cells changed slightly from cells harvested during the initial growth and from those cells harvested from the stationary phase. There was a shift in the shape of the cells from a typical coccal structure to more elongated forms. Also on examination under microscope it was found that the cells tend to form clusters, which could be reason for variable results obtained in the various cell counts as illustrated in below Figure 2.6 and Figure 2.7. The tendency to form clusters affects the OD600 readings and this was minimised by shaking the flasks rigorously before readings were taken to break up bigger clusters.

Microscopic view of the cultures in glycerol during the initial growth phase and the stationary phase are shown below. An Olympus LH50A microscope fitted with a JVC camera was used to obtain these photos.

Figure 2.6: Initial growth phase showing individual cells

Figure 2.7: Cells in stationary phase where cell clumping is very visible

Growth studies were performed on *R. opacus* growing on glycerol as the sole carbon source. This was carried out to optimize the concentration of glycerol required for optimum growth and to determine the maximum surfactant production. All the growth studies were carried out in duplicate flasks. 100mL of Medium B were taken in 250mL Erlenmeyer flasks and autoclaved at 110°C with 10 minute sterilisation time. Various concentrations (1%, 2%, 4%, 6% and 8%) of glycerol were added to the flask on a weight-by-weight basis. Prior to seeding the flasks, the optical density measurements of the medium were taken as zero for later measurements. Each of the two flasks for the five sets of percentages was seeded with 1mL of the stock culture.

The OD600 values were taken immediately after seeding the cultures to get the first set of OD600 values for 0^{th} hour. From this point the OD600 values were taken at regular intervals using the spectrophotometer to monitor cell growth. To analyse the rate of substrate utilization, samples taken each time a spectrophotometer reading was recorded were stored at -30°C to be analysed together once the growth study was completed. The utilization of carbon source glycerol was measured using a High Performance Liquid Chromatography (Dionex thermostatted column compartment TCC-100 fitted with Dionex GS50 gradient pump and ED50 electrochemical detector). The auto-sampler used in these experiments is Famos by LC packing, UK.

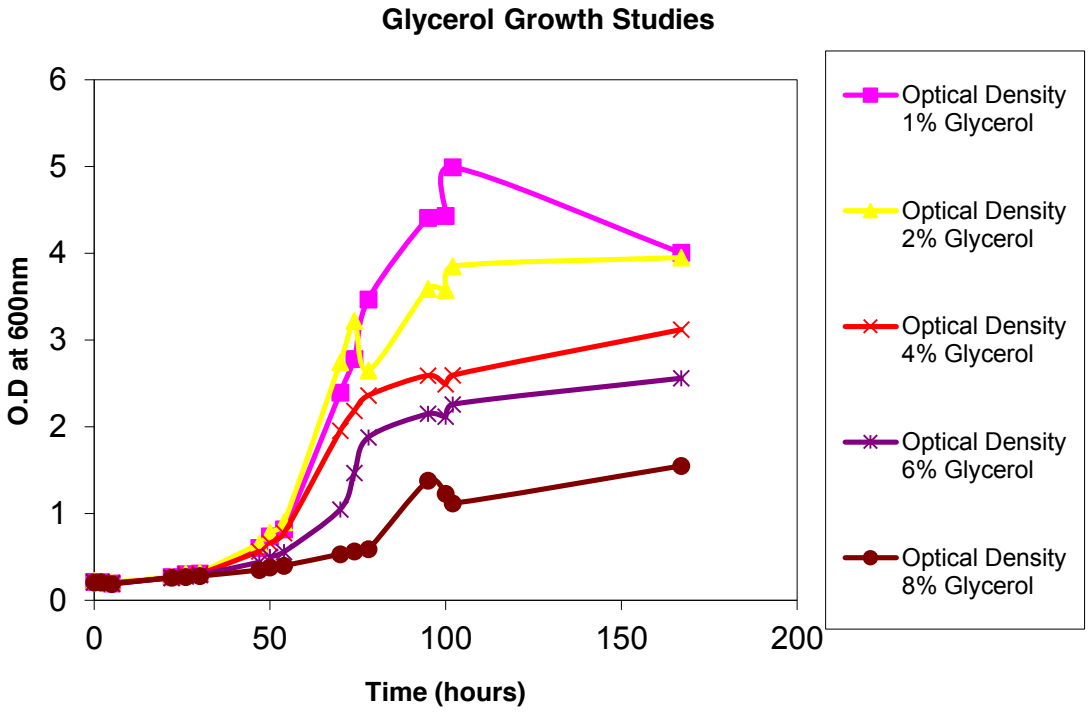


Figure 2.8: Graph of growth studies of *Rhodococcus opacu*s in various percentages of glycerol

The adaptation in the various solvents has been successfully carried out for pure glycerol. Other adaptation cultures in crude glycerol and methanol has been carried out successfully and more than six generations of each culture was obtained. Further growth studies need to be conducted on cells grown on crude glycerol to observe the level of surfactant production. Also according to Ciapina *et al*. (2006), bacteria theoretically must produce extra-cellular surfactants in the presence of water-soluble carbon sources but this was found to be untrue as illustrated in the following chapters.

Along with these sets of flask a similar study was conducted simultaneously for R. *opacus* cells growing on glucose as the sole carbon source. The decrease in glucose level was monitored by using a Lightwave spectrophotometer (Lightwave, UK) every time an OD600 reading was taken.

The maximum growth rate μ was calculated by taking 3 points from the steepest part of the bacterial growth curve. These values were then converted into the corresponding cell number using the correlation obtained between the turbidity and the haemocytometer cell counts. The 3 points were plotted on a semi log graph with the cell numbers represented on the Y-axis and the time in hours on the X-axis. Following this a regression fit is made and the maximum growth rate μ was obtained. The highest OD600 value obtained was taken as the maximum biomass and converted into the cell number. The above procedure was done to both the growth studies. The results obtained so far are shown in the section below.

2.4.1 Data Analysis Using HPLC:

On completion of the growth studies, it was possible to calculate the increase in the cell count by the recorded OD600 values. In order to calculate the rate of substrate utilization, it is essential that the decrease in the amount of carbon source - glycerol is calculated. This was done using a High Performance Liquid Chromatography column (HPLC).

In order to use the HPLC to detect the decrease in the concentration of the glycerol in the medium, previously mentioned samples were stored as and when the OD600 values were taken and stored at -30°C. This was to prevent the cells from growing during storage period. A separate set of known standard solution of glycerol were prepared to be used in each of the runs and a quality control solution was also prepared based on which the accuracy of the results were determined. Before samples were loaded into the glass vials, the samples were centrifuged at 4500 g^{-1} for 90 minutes to make sure that all the cells and debris settle down at the bottom. Depending on the percentage of glycerol sample being analysed the dilution factor was decided. The vials

were loaded with the supernatant after centrifugation and diluted with the appropriate amount of deionised water. After successfully loading all the samples into the glass vials they are sealed and arranged sequentially onto the auto-sampler.

After loading the samples into the auto-sampler, the various parameters in the HPLC operating software were modified and the run is initiated. Following the run, the individual peaks for each of the samples were analysed. Depending on the conversion factor that is obtained from the standard graph plotted using peak data from the known concentrations of the standards and depending on the dilution factors, the molar concentration of the glycerol was calculated.

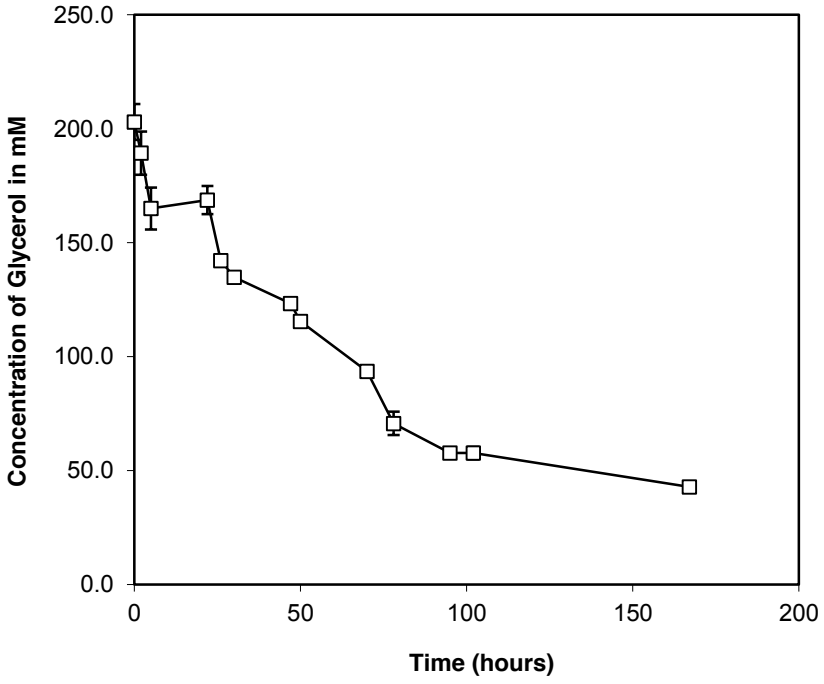


Figure 2.9: Decrease in concentration of glycerol during growth of R. *opacus* on 1% (w/w) of pure Glycerol

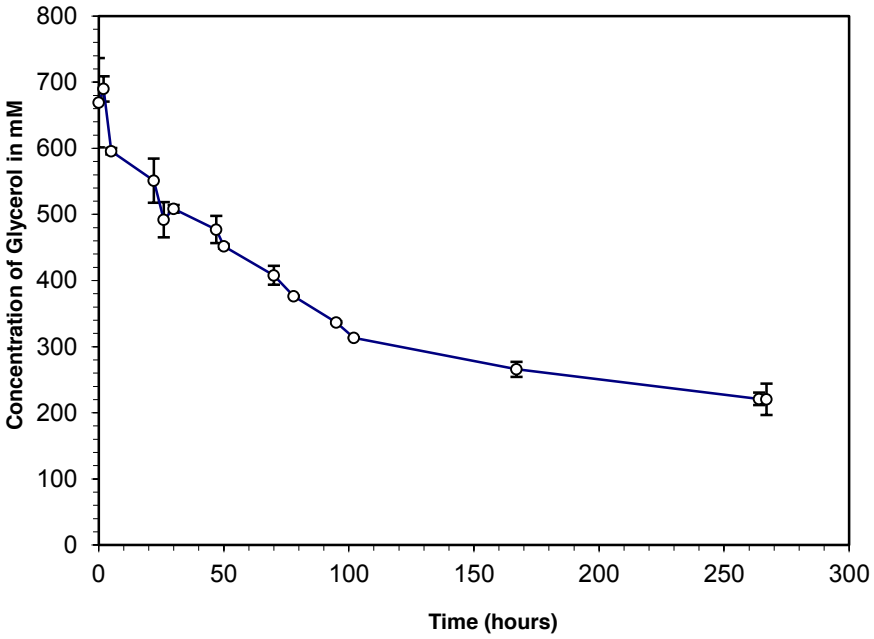


Figure 2.10: Decrease in concentration of glycerol during growth of R. *opacus* on 4% (w/w) of pure glycerol

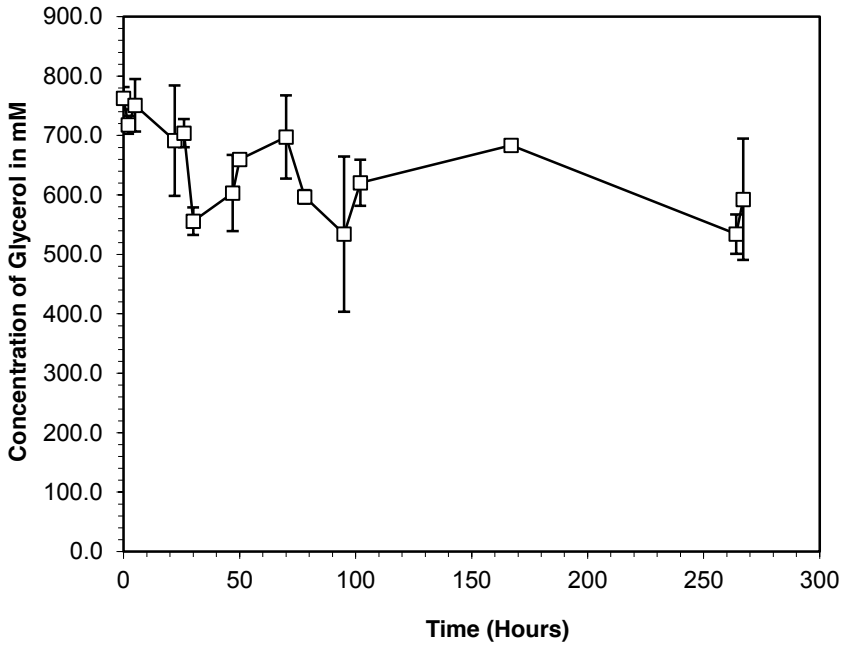


Figure 2.11: Concentration of glycerol during growth of R. *opacus* on 6% (w/w) pure glycerol

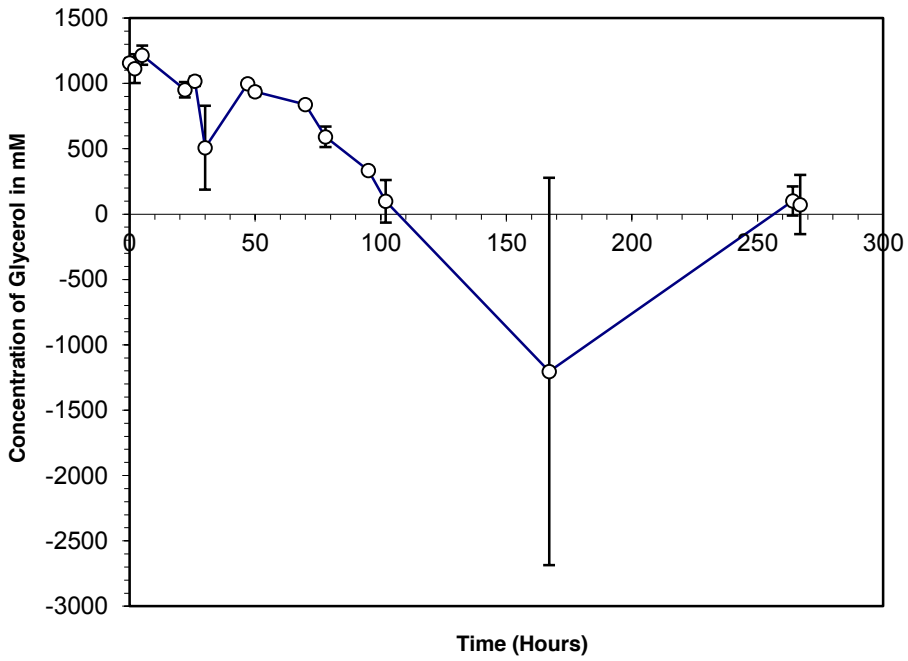


Figure 2.12: Concentration of glycerol during growth of R. *opacus* on 8% (w/w) pure glycerol

All the experiments were carried out in duplicate to avoid the errors that could occur due to contamination or instrumental error. In spite of efforts taken the 2% glycerol study samples could not be analysed due to very high initial glycerol concentrations that were obtained after the first run. During the experimental setup all the flasks were added with equal volume of glycerol and were weighed out as accurately as possible. Although equal amount of glycerol was weighed out, the results obtained after the HPLC run invariably showed higher initial concentrations of glycerol than originally added. This was thought to occur during the process of autoclaving, which was done at 121˚C with a sterilisation time of 15 minutes. It was expected that there would be preferential evaporation of the water that would account for the slightly increased concentration of glycerol.

From the various results it can be seen that the concentration of glycerol steadily decreased in the cultures that were grown on 1% and 4% glycerol samples before the cells reach the stationary phase. This could be because either the glycerol concentration was too low for the organisms to grow on or there is a chance of production of certain toxic waste products like organic acids, which was detected by the HPLC to a certain extent. For the 6% and 8% samples the glycerol concentration varied a lot more than the previous two percentages. In both of them after steady decline there was a steep climb in the glycerol concentration. The only explanation for this occurrence is that some of the cells disintegrate due to the higher glycerol concentrations and hence releasing their cell components into the culture medium. This is the only plausible explanation to the sudden increase in glycerol concentration since there is possibility of a co-metabolic growth since carbon source is strictly maintained only as glycerol and a minimal salt media is used in all the experiments.

In general the trend of change of concentration of glycerol over the course of the growth of the organism is to decrease until the glycerol concentrations become too low or due to the accumulation of some other toxic metabolites in the culture medium.

Chapter 3: Optimisation of Growth Parameters

This chapter describes three aspects of the optimisation process which are optimum operating temperature, nitrogen source and pH range. These optimisation experiments have been carried out in order to ascertain the operating parameters that would produce the best possible growth of target organisms that would be directly linked to the biosurfactant production levels. Along with these optimisations, this chapter also deals with experiments that were carried out to assess the effect of increasing NaCl concentrations in the culture media and how this would impact the growth of the two bacterial strains.

3.1 Identification of Optimum Temperature:

Chapter 2 described the first set of growth studies that were carried out to investigate the growth of *Rhodococcus opacus* in glycerol and methanol (Figure 2.7 and Figure 2.8). Second sets of experiments were carried out to calculate the optimum temperature range for the *Rhodococcus opacus* strain. Duplicate flasks were used for all the different temperatures. The glycerol concentration was maintained at 3% (w/w) for the three set of temperatures 20˚C, 23˚C and 25˚C. Based on results from Chapter 2, which looked at the rate of utilisation of Glycerol at various concentrations, it was decided that an optimum concentration of 3% would be chosen and maintained at this level for future experiments involving glycerol as the carbon source. Glycerol was added weight by weight to each of the flasks. All the flasks were shaken at 130 RPM. After taking the OD600 values for the medium after sterilisation, all the flasks were

seeded with 1mL of the stock culture. The stock culture used for this study was the 15th generation of R. *opacus* growing on glycerol as sole carbon source.

The experiment was conducted simultaneously using three different shakers maintained at the three different temperatures. Apart from the temperatures, all the other conditions such as substrate concentration and shaker speed were maintained constant. OD600 values were taken at regular intervals to obtain points on all phases of cell growth. The results are presented below in Figure 3.1.

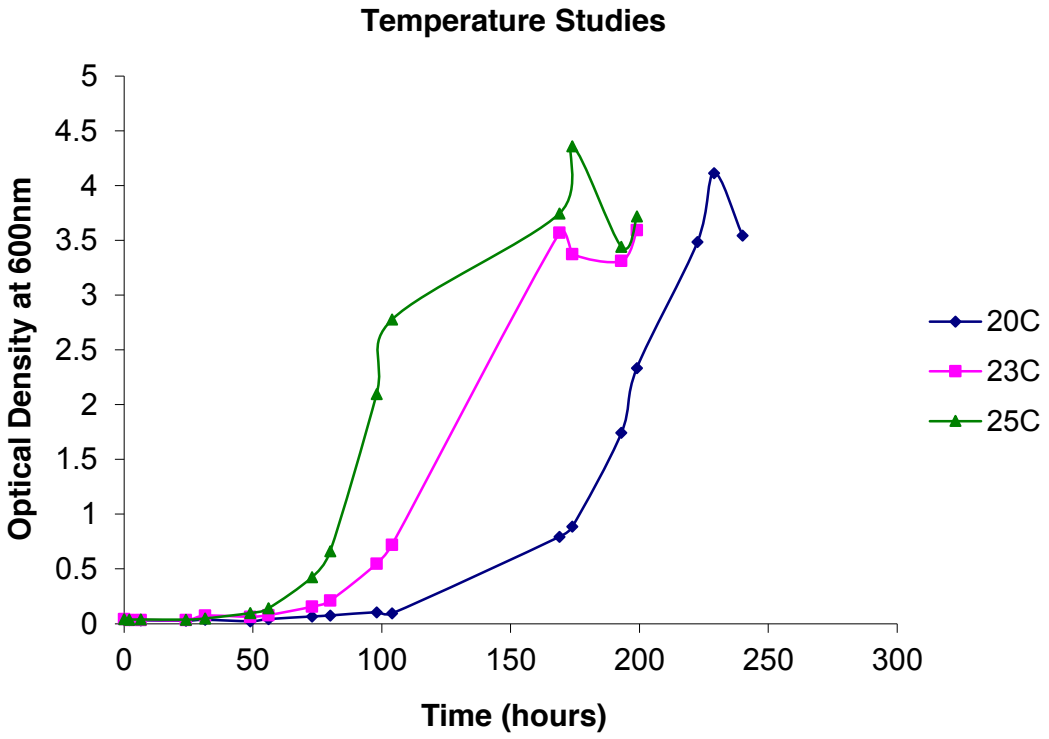


Figure 3.1 Graph of Growth study on *R. opacus* in 3% glycerol to obtain optimum temperature

From Figure 3.1 it can be inferred that while all three temperatures would achieve similar levels of growth, the shortest lag phase would be achieved at a temperature of 25˚C.

3.2 Identification of Optimum Nitrogen Source:

In order to test the effect of the various nitrogen sources on the growth of the bacteria growth, studies were performed on both *R*. *opacus* and *R*. *ruber* in order to optimise the nitrogen source required for maximum growth and to determine the maximum surfactant production. Studies have shown that higher biomass concentrations achieved as a result of medium optimisation would indicate greater levels of substrate utilisation as well as elicit increased production of secondary metabolites (Karpenko *et al*., 2006). All the experiments were carried out in duplicate. 100mL of Medium B was placed in 250mL Erlenmeyer flasks and autoclaved at 121 °C for 15 minutes along with 1 mL n-hexadecane. All the other prevailing conditions were maintained as constant as possible with the only variations being the changes in the composition of medium with respect to the chosen nitrogen sources namely ammonium sulphate, ammonium nitrate, sodium nitrate and potassium nitrate.

Prior to seeding the flasks with the inoculum, the optical density measurements of the medium were taken so that the suitable correction factors can be incorporated into the optical density values obtained during the course of the experiment. Each of the two flasks for the four nitrogen sources were seeded with 1mL of the stock culture. Care was taken to ensure that the stock culture was in the exponential phase of growth for the particular strain. The OD600 values were taken immediately after seeding the cultures to get the first set of OD600 values for the 0^{th} hour. From this point onwards the OD600 values were recorded at regular intervals using the spectrophotometer to get data on all phases of the bacterial growth curve. In order to quantify the amount of surfactant that is being produced, every time a sample was taken to measure optical density, another

sample was withdrawn and stored at -30 °C to be analysed together once the growth study was completed. The quantification of surfactant was measured using the Phenol-Sulphuric acid assay for carbohydrates.

The above procedure was repeated in a similar manner for *R*. *ruber*. After the first set of growth studies carried out in order to optimize the nitrogen source, another set of experiment was carried out in order to optimize the concentration of the nitrogen source, which was chosen for further study. The conditions for this experiment are almost identical as the previous experiment. All the growth conditions such as temperature and shaking speed were kept constant with only the concentration of the nitrogen source changed. Three concentrations of sodium nitrate were used namely 0.25 g/l, 0.5g/l and 1.0g/l. Similar to the previous experiment growth was monitored spectrophotometrically and samples stored at -30°C periodically for further analysis.

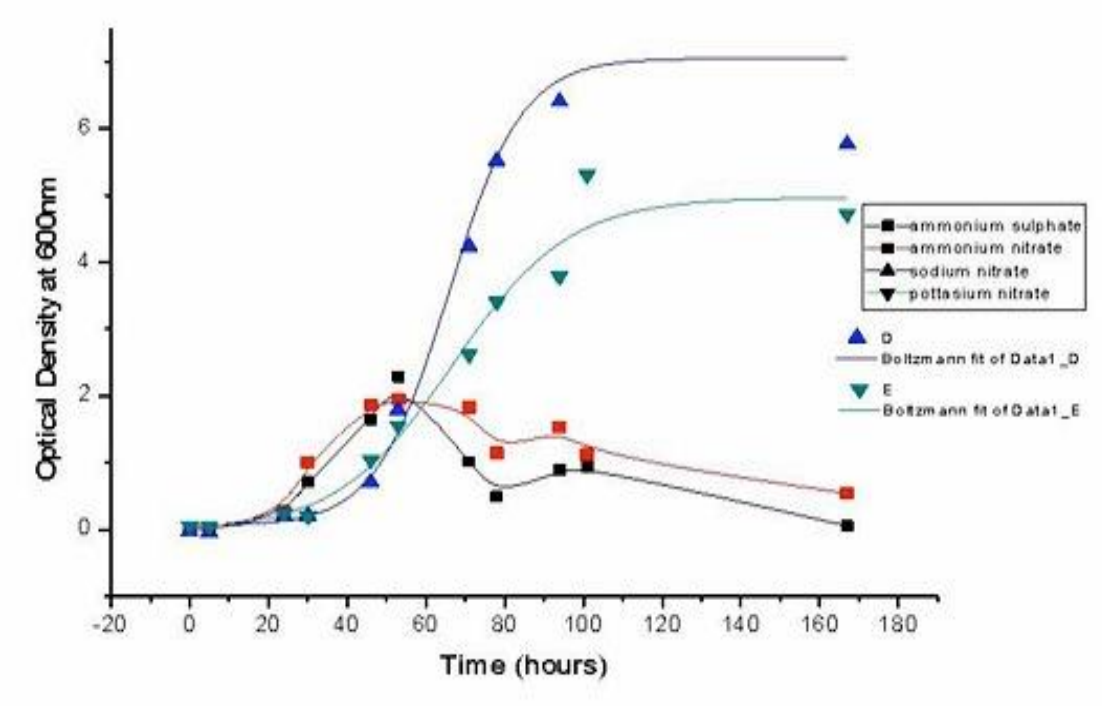


Figure 3.2: Growth of *R*. *opacus* on the various nitrogen sources

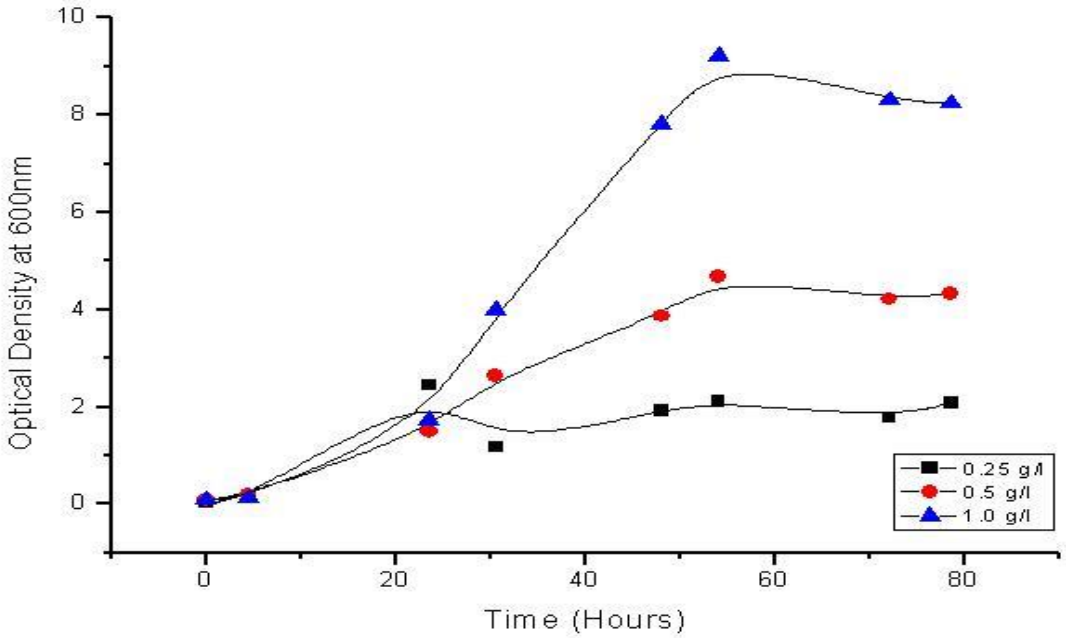


Figure 3.3: Growth of *R. opacus* on the varying concentrations of sodium nitrate

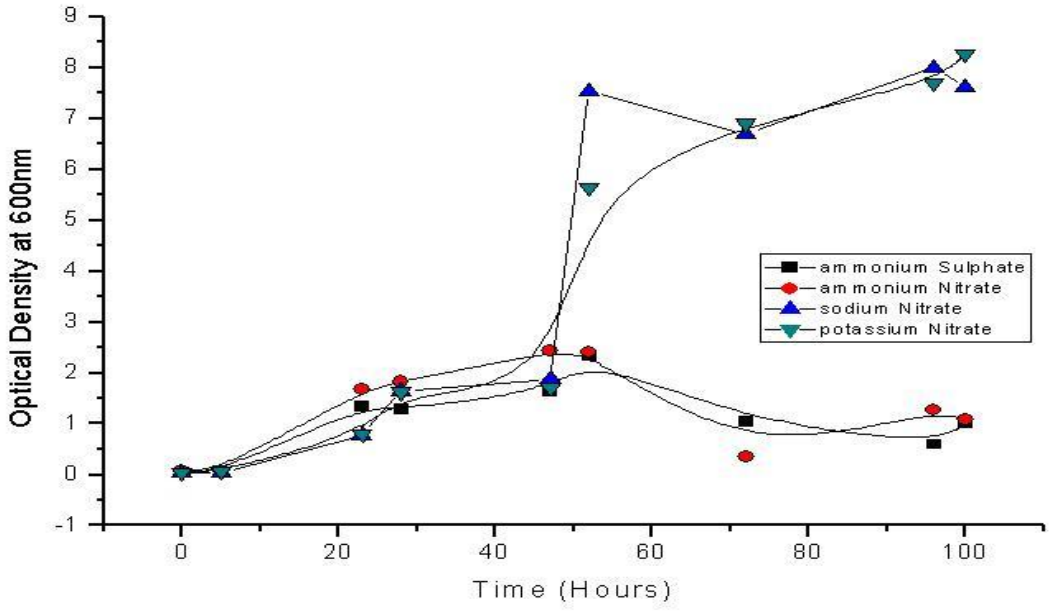


Figure 3.4: Growth of *R. ruber* on different nitrogen sources

Figure 3.2, 3.3 and 3.4 above represent the data obtained for growth study experiments that were carried out in order to determine the optimum source of nitrogen. As found by other researchers (Moussa *et al*., 2006; Abouseoud *et al*., 2008), medium optimisation plays an important role in maximizing the production of surfactants. This is crucial in order to make the production of biosurfactants as economical as possible and thus would enable biosurfactants to compete in the mark*et al*ongside chemical surfactants. It was proposed that with the development of economical engineering processes and the use of low cost raw materials, the cost of production of biosurfactants could be reduced nearly by 50% (Moussa *et al*., 2006), which could also reduce the cost of waste treatment.

Based on the data obtained from the experiments carried out with *R*. *opacus* and *R*. *ruber* using the four nitrogen sources, it was found that there was a marked difference in the rate of growth and hydrocarbon assimilation. Both the strains of *Rhodococcus* exhibited higher growth in the nitrates rather than the ammonium salts (Figure 3.2, Figure 3.4). The next step would be to analyse the secondary metabolites that are being produced in order to map the metabolic pathway that the bacteria follow in order to degrade hydrocarbons. It was proposed to use a mass spectroscopy combined with a gas chromatography in order to determine the structure of the surfactants produced which aid in the uptake of hydrophilic compounds.

Using data from the growth experiments, a growth curve has been established for the two strains using n-hexadecane as the sole carbon source. From the data the maximum growth rate μ_{max} for bacteria under each of the different growth conditions were calculated along with the maximum biomass obtained using the method described in Chapter 2.

Another set of growth studies using 1.5 ml n- hexadecane as the sole carbon source in the presence of various percentages of NaCl was carried out to observe the growth of *Rhodococcus opacus* in the presence of salt. These experiments were carried out to obtain data on the growth of this species in the increasing concentrations of NaCl, which is often found at petroleum reservoirs. A series of experiments were carried to determine the extent the different concentrations of salt would have on the total growth obtained. A range of salt concentrations from 1g/l to 10g/l of NaCl was tried out. All experiments were carried out in duplicate and maintained at 25°C while being shaken at 130RPM. This is represented by data presented in Figure 3.5 and Figure 3.6.

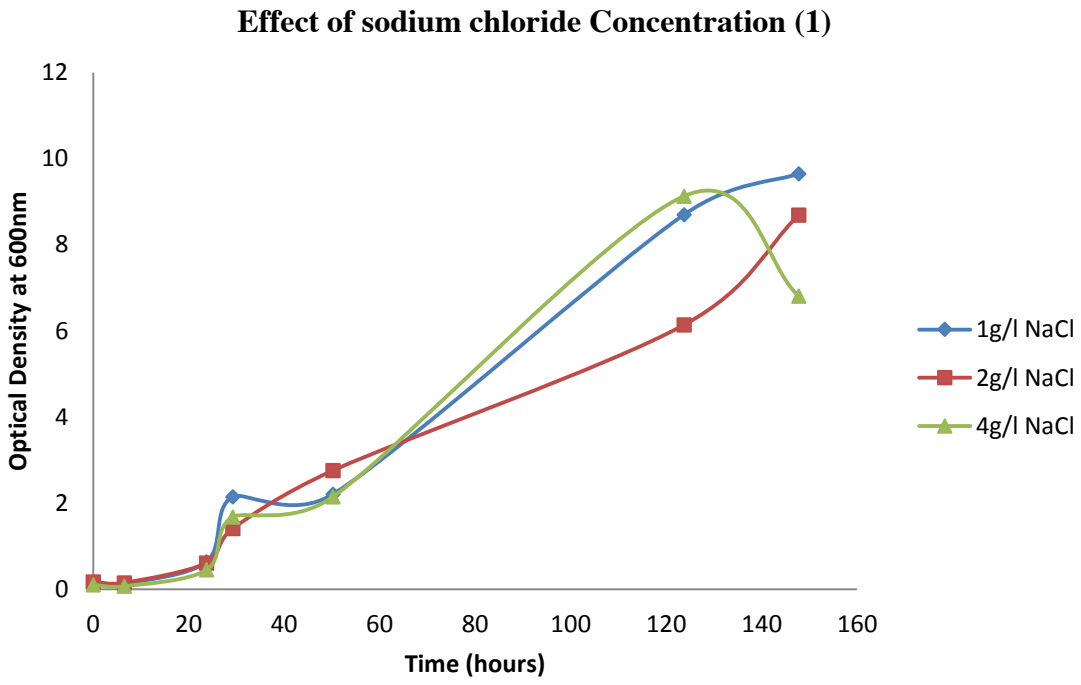


Figure 3.5: Effect of sodium chloride concentration on growth of *R. opacus*

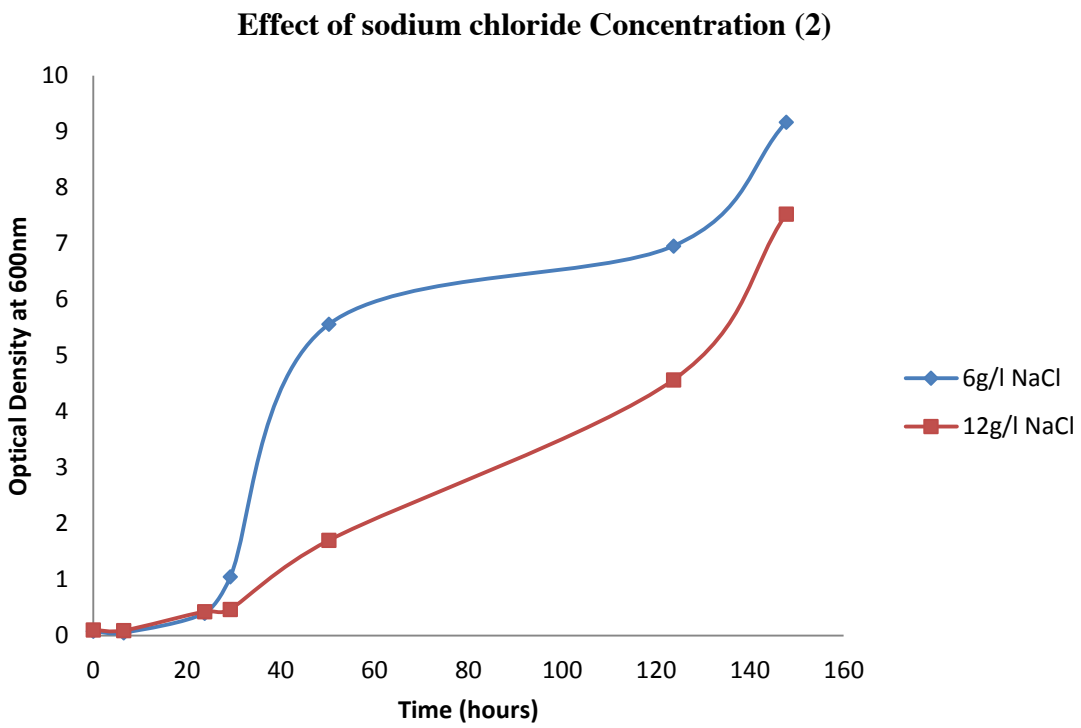


Figure 3.6: Effect of sodium chloride concentration on growth of *R. opacus*

The data obtained from the experiments carried out with varying salt concentrations as presented in Figures 3.5 and 3.6, indicate that there isn't considerable effect on the growth of the bacteria. In order to use biosurfactants for in-situ treatments, the ability to grow in high salt concentrations would be beneficial since many sites are situated in highly saline areas. From the growth curves it is evident that there is a fair degree of growth in almost all the NaCl concentrations that the bacteria were grown in. In order to test the variances within the different growth curves, an ANOVA was performed and the results are as follows:

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.142	2	.571	.042	.959
Within Groups	243.698	18	13.539		
Total	244.840	20			

Table 3.1: ANOVA on *R. opacus* grown on various salt concentrations 1g/l, 2g/l, 4g/l

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.016	1	5.016	.441	.519
Within Groups	136.586	12	11.382		
Total	141.602	13			

Table 3.2: ANOVA on *R. opacus* grown on various salt concentrations 6g/l and 12g/l

From the ANOVA results it can be seen that significance values of 0.959 and 0.519 were obtained which suggest that there is a high likelihood that the various salt concentrations have little effect on the growth of the bacterium. The ability of *Rhodococcus opacus* to grow on fairly high concentrations of NaCl would make *Rhodococcus opacus* ideally suited for use in environments that are otherwise hostile to most other bacteria used in bioremediation. Salt concentration and pH are the main factors that make use of bacteria in field trials and in-situ applications very difficult but this would have negligible effect on this particular strain of bacteria.

The maximum growth rate of R. *opacus*, $\mu_{max,}$ was calculated as described in the previous chapter.

3.3 pH Studies and Total Alkalinity:

Experiments were carried out with *R. opacus* and *R. ruber* in order to monitor the changes in pH of the medium during the course of growth on n-hexadecane. All the media for the experiments were prepared and autoclaved at 121 °C for 15 minutes. The inoculation was carried out with the stock culture in the exponential phase of growth and periodically samples were withdrawn to observe growth of bacterial cells spectrophotometrically as well to measure the pH. pH measurements were carried out using Hanna instruments: pH 210 Microprocessor pH meter. Before any measurement was made using the pH meter, the instrument was calibrated using pH 4 and pH 7 buffers prepared freshly and stored at 4 °C.

Another set of experiments were carried out to ascertain the pH ranges that the bacteria were capable of adapting to and thriving on. As above all media were inoculated with stock culture in the exponential phase of growth. All the experiments were carried out in duplicate unless stated otherwise. A pH range of 3 to 13 was tested. Samples were withdrawn at regular periods to measure the growth spectrophotometrically and to carry out pH measurements.

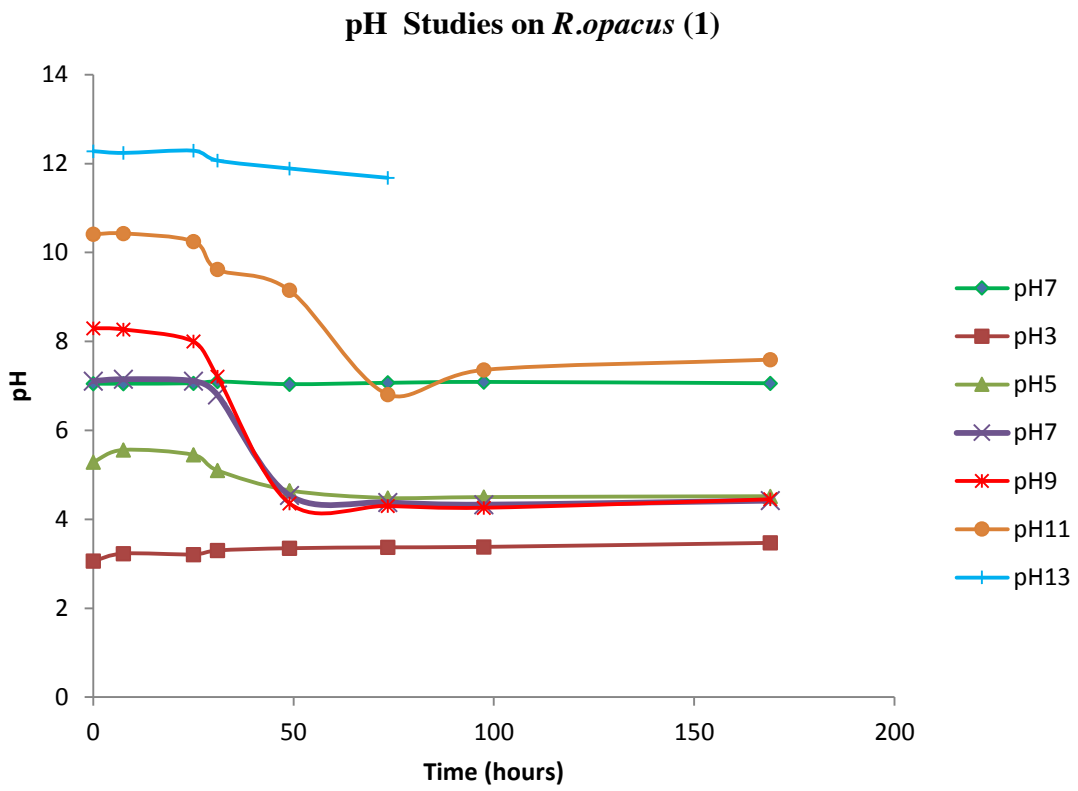


Figure 3.7: Variation in pH during *R. opacus* growth in various initial pH

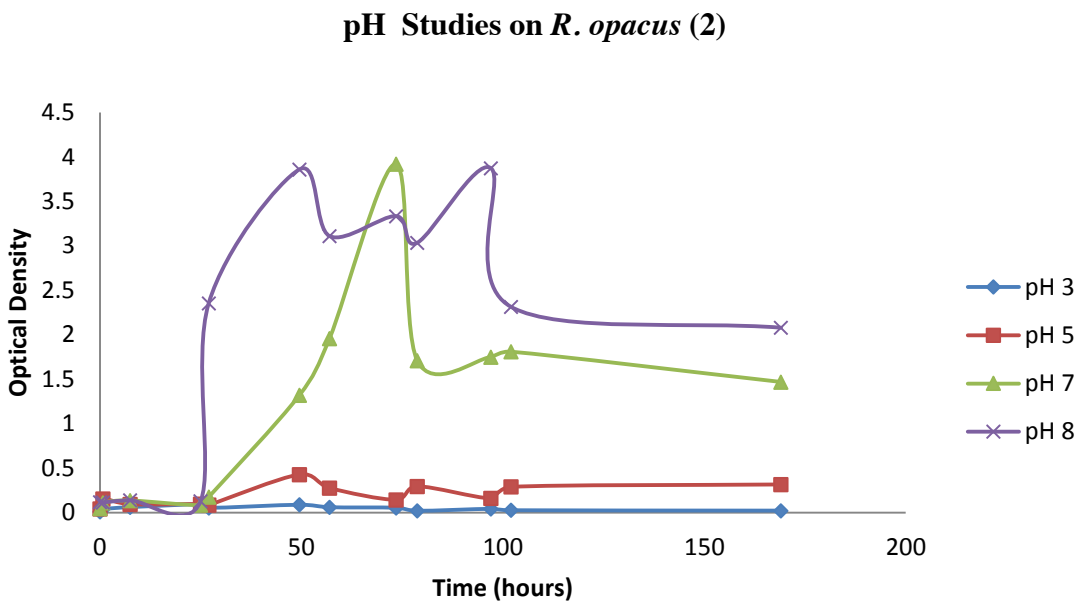


Figure 3.8: Growth of *R. opacus* in various initial pH

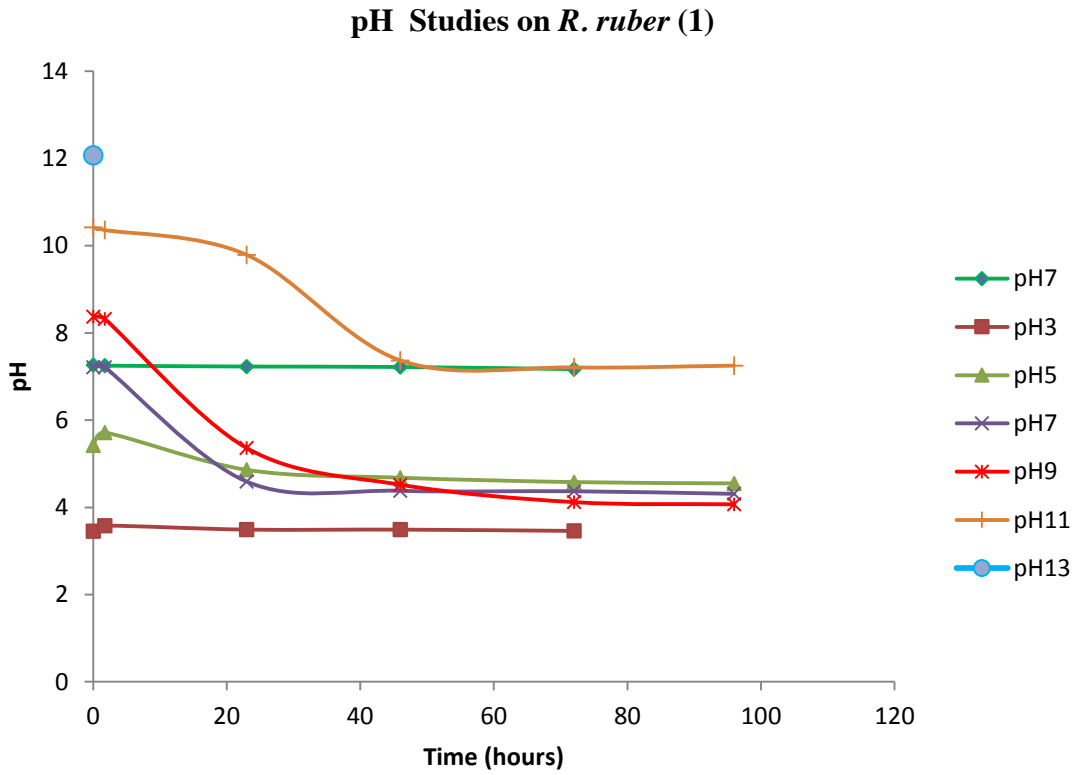


Figure 3.9: Variation in pH during *R. ruber* growth in various initial pH

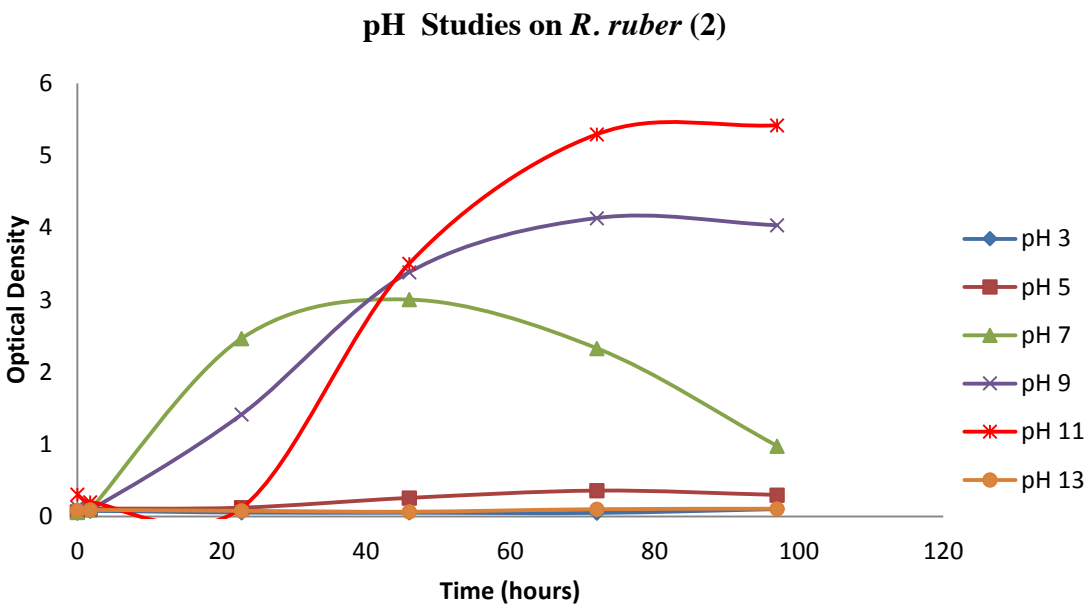


Figure 3.10: Growth of *R. ruber* in various initial pH

In order to understand the effect of the initial pH on the overall growth potential of the bacterial strains, further experiments were carried out in various pH ranges as represented in Figures 3.7, Figure 3.8, Figure 3.9 and Figure 3.10. pH ranges of 3 – 13 were tested. From the data and the graphs it can be seen that overall a drop in pH is observed during the course of bacterial growth with the exception of pH 3 and pH 13 where there is negligible growth or pH variation. This is observed uniformly in both the bacterial strains. This clearly shows that the bacteria have a preferred pH range under which there tends to be maximum growth show by the increase in optical density values and corresponding increase in biomass. In spite of the drastic differences in the observed pH, the changes in the observed biomass values are not as extreme in the variation as might be expected for such a range of pH. In order to understand the variation expressed within the pH ranges tested in terms of expressed biomass and growth potential, an ANOVA (Analysis of Variance) test was carried out.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	174.562	4	43.640	2.175	.084
Within Groups	1103.472	55	20.063		
Total	1278.033	59			

Table 3.3: ANOVA on *R. opacus* grown on various initial pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	36.895	5	7.379	3.733	.010
Within Groups	59.305	30	1.977		
Total	96.200	35			

Table 3.4: ANOVA on *R. ruber* grown on various initial pH

From the ANOVA test results, a significance of 0.084 on the *R. opacus* experiment means that it is above the threshold value of 5% significance level and hence the null hypothesis of the ANOVA is rejected. What this effectively means is that although there is considerable difference in the pH variation the growth potential of *R. opacus* has not be considerably affected. This effectively reinforces the robustness of the *R. opacus* strain. The bacterial has been capable of manipulating the surrounding environment successfully and has been able to show promising growth in at least a pH range of 5 – 11. With further adaptation it would be definitely possible to enhance the survival rate of *R. opacus* in more inhospitable environments.

On the other hand the growth of *R. ruber* is considerably different from that of *R. opacus*. The growth of *R. ruber* on the various initial pH seems to be more erratic and quite dependent on the initial pH that it has been grown on. From the ANOVA test that was carried out, a significance of 0.010 on the *R. opacus* experiment means that it is below the threshold value of 5% significance level and hence the null hypothesis of the ANOVA is accepted. This would mean that there is considerable difference in the growth potential depending on the initial pH of the growth media. The *R. ruber* has not

been as successful in adjusting to extreme pH conditions and this would have to be taken into consideration during the design and operation of the larger scale experiments and the membrane reactor.

Total alkalinity of the medium with each of the four nitrogen sources were carried out to ascertain the alkalinity of the medium. Alkalinity of water is its acid – neutralising capacity. It is a measure of the buffering capacity of water that is its ability to withstand pH changes (E.M. McEvoy, 2006). These experiments have carried out in order to investigate if the difference in the growth rate of the organisms in the media with different nitrogen sources is a nutritional deficiency or linked to the buffering capacity of the media.

The experiment was carried out by first transferring 50mL of the medium into a conical flask or a beaker. A 10mL burette is filled with 0.1M HCL solution and fixed on a burette stand. Titration was carried out until pH 4.5 was achieved. The pH was monitored by using a pH meter that was placed inside the beaker containing the medium. The total alkalinity was calculated using the formula:

Alkalinity, mg $CaCO_3$/ L = (A x N x 50000) / ml sample

A = ml of standard acid used

N = normality of standard acid used

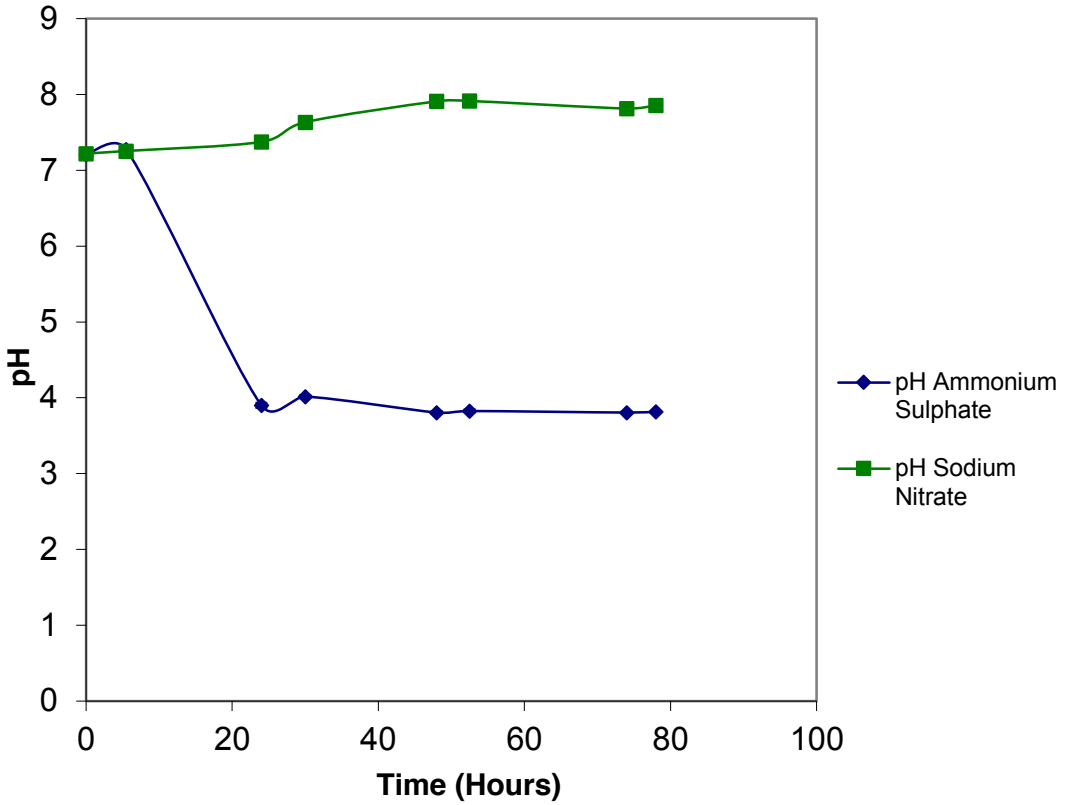


Figure 3.11: pH versus Time for *R. opacus* on ammonium sulphate and sodium nitrate

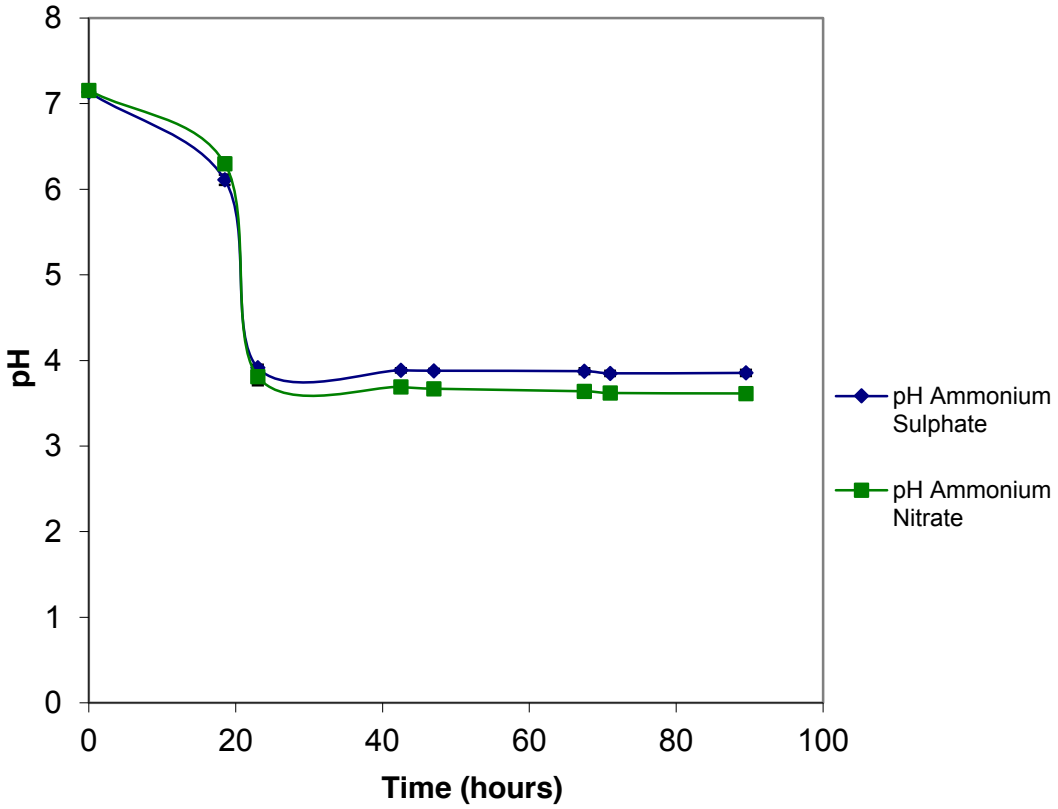


Figure 3.12**:** pH Vs Time for *R. opacus* on ammonium sulphate and ammonium nitrate

After the above two experiments were conducted, it was concluded that the drop in pH occurred only in the ammonium salts. Due to the effect of ammonium sulphate and ammonium nitrate on the growth of R. *opacus* it was decided to check the alkalinity of the Medium B with all the four nitrogen sources.

Sample ID	**Alkalinity**
Medium B with Ammonium Sulphate	190 mg $CaCO_3$ / L
Medium B with Ammonium Nitrate	200 mg $CaCO_3$ / L
Medium B with Sodium Nitrate	200 mg $CaCO_3$ / L
Medium B with Potassium Nitrate	210 mg $CaCO_3$ / L

Table 3.1: Alkalinity of Medium B with the four nitrogen sources

From the observed differences in the growth of R. *opacus* on the different nitrogen sources, it was decided to monitor the change in pH of the medium during the course of the bacterial growth. This was performed in order to ascertain whether the drop in pH was the reason behind the poor growth of R. *opacus* on the ammonium salts. For the first experiment, R. *opacus* was grown on ammonium sulphate and sodium nitrate and for the second experiment R. *opacus* was grown on ammonium sulphate and ammonium nitrate. These two experiments were conducted in order to prove that the drop in pH was due to the ammonium salts rather than the nitrates. This in fact was proved in the course of the two experiments that were carried out.

After the above two experiments were conducted it was concluded that the drop in pH occurred only in the ammonium salts. Due to the effect of ammonium sulphate

and ammonium nitrate on the growth of R. *opacus* it was decided to check the alkalinity of the Medium B with all the four nitrogen sources.

Alkalinity of medium had not been found to be considerably different for each of the four nitrogen sources. This would suggest that, all the four salts have almost the same effect on the buffering capability of Medium B. Therefore it is probably the cell metabolism of *Rhodococcus opacus* in the various salts that results in the drop in pH ultimately causing the poor growth in ammonium sulphate and ammonium nitrate. Clearly the nature of the nitrogen source has an effect on the bacterial growth due to the change in the pH, which can make it inhospitable for further growth.

Chapter 4: Biosurfactant Characterisation

This chapter deals with the characteristics of the surfactant that is being produced by *Rhodococcus opacus* and *Rhodococcus ruber*. The tests that are described in this method include the emulsification ability, the degree of adhesion to hydrocarbons and surface tension measurements, which are important factors that need to be considered when testing the effectiveness of a biosurfactant. Chapter 4 also describes in detail the method that has been utilized to quantify the biosurfactants being produced while also studying the effect of various nitrogen sources.

4.1 Emulsification Activity of Biosurfactant:

Emulsifying capability is one of the characteristics of biosurfactants apart from the lowering of the surface tension and the interfacial tension (Ciapina *et al*, 2006). Ciapina *et al* (2006) developed a modified form of the original emulsification index measurements done by Cooper & Goldenberg (1987). In the original experiment, the emulsifier activity was measured by adding 6mL of kerosene to 4mL of the aqueous sample. This mixture was vortexed at high speed for 2 minutes and then allowed to settle. Height measurements of the emulsified layer were made 24 hours later. The emulsion index (E_{24}) is the height of the emulsion layer, divided by the total height multiplied by 100 (Cooper & Goldenberg, 1987).

There have been many modifications of the original method. Mody (2006) used a modified form of the Cooper & Goldenberg method where the kerosene was replaced by other hydrocarbons and the height measurements made even after 24 hours. The experiments carried out in this project are a slight modification of the original Cooper &

Goldenberg method. In this method the quantity of kerosene and the aqueous phase have been reduced to 3mL and 2mL respectively. Samples were taken from the liquid cultures of *Rhodococcus opacus* and *Rhodococcus ruber* grown on various carbon substrates. Different carbon sources were employed in order to ascertain their effect on the emulsification capability of the biosurfactants produced. All the samples were centrifuged at 4500g for 90 minutes and the supernatants were collected. In a clean test tube, 2mL of the supernatant of the bacterial culture broth was added along with 3mL of Kerosene. The above was repeated for each of the remaining percentages. All the test tubes were vortexed for 1 minute at high speed and then kept stationary. The height measurements were taken 24 hours later. The experiments were carried out using Kerosene and n-hexadecane to investigate any differences. All the emulsification tests were carried out in duplicates and the average values are presented in the results below.

The emulsification index values obtained for the various samples are presented below in tables 4.1, 4.2 and 4.3.

Sample	Height of emulsion layer in mm	Total height	E_{24}
Control	0	35	0
1% Glycerol	1	36	2.8
2% Glycerol	0	36	0
4% Glycerol	4	36	11
6% Glycerol	3.5	34.5	10
8% Glycerol	0	35	0

Table 4.1: E_{24} values obtained on R. *opacus* grown on varying concentrations of glycerol

Sample	Height of emulsion layer in mm	Total height	E_{24}
Control	0	33	0
R. *opacus* in Glucose	6	33	18.18
R. *opacus* in Methanol	3	34	8.823
R. *opacus* in Glycerol	9	33	27.27

Table 4.2: E_{24} values of R. *opacus* grown on various carbon sources

Sample	Height of emulsion layer in mm	Total height	E_{24}
Control	0	33	0
R. *opacus* in n-hexadecane (culture medium)	12	33	36.36
R. *ruber* in n-hexadecane (culture medium)	11	34	32.35
R. *opacus* in n-hexadecane (supernatant)	8	33	24.24
R. *ruber* in n-hexadecane (Supernatant)	10.5	35	30
R. *opacus* in Kerosene (culture medium)	11	34	32.35
R. *ruber* in Kerosene (culture medium)	13	36	36.11
R. *opacus* in Kerosene (Supernatant)	9	33	27.27
R. *ruber* in Kerosene (Supernatant)	12	34	35.29

Table 4.3: E_{24} values for R. *opacus* and R. *ruber* in n-hexadecane and kerosene using cell culture and supernatant after centrifugation

In the first experiment that was carried out using media obtained from *R. opacus* grown in various concentrations of glycerol, considering relevant literature studies the emulsification achieved as represented in Table 4.1 was found not to be very high compared to similar studies. This indicates that either the surfactant produced is of smaller quantity and the emulsifying activity is not pronounced or the production of surfactant has not been increased by the presence of a hydrophilic carbon source.

On the other hand the second experiment conducted has a marked difference in the emulsion percentage as represented in Table 4.2. This could be because the increase in emulsion activity is attributed to the possibility that the surfactants are cell bound.

In an experiment carried out by Ciapina (2006), it was experimentally proven that surfactants were cell bound. There was an increase in the amount of surfactants when the aqueous medium containing the cells was subjected to sonification. In order for a biosurfactant to have commercial value, it must be produced in large quantities and preferably secreted into the medium to facilitate easy recovery (Cooper and Goldenberg, 1987).

The initial data obtained from the use of glycerol, glucose and methanol as carbon sources confirm that the production of surfactants is initiated with the presence of alkanes in the growth medium. As seen in the subsequent experiments, surfactant activity increased with the use of n-hexadecane as the sole carbon source as is evident from Table 4.3. In order to verify if the surfactants were bound to the cell wall or released into the culture media, the various emulsification assays were carried out both with the culture media as well as the supernatant obtained by sonification followed by centrifugation.

There was a marked difference in the emulsification index values obtained for *R. opacus* when carried out using culture media and the supernatant whereas such was not seen when *R. ruber* was employed. This clearly indicates that the surfactants are cell bound in *Rhodococcus opacus* whereas with *Rhodococcus ruber* they are released into the growth medium. This could mean that the method of hydrocarbon assimilation could be different between these closely related bacterial strains. This is important since it would affect the way these two bacterial strains would interact when cultured together which will be discussed in the following chapter. The degree of emulsification is important since it gives an estimate regarding the potency of the surfactant that is being produced by the bacteria.

4.2 Bacterial Adhesion to Hydrocarbons (BATH ASSAY):

There are currently many methods used for studying the hydrophobic interactions of cells but there is no single method that adequately explains the cell-surface hydrophobicity since the conditions employed are influenced by the hydrophobic interactions and also various non-hydrophobic effects interfere with the analysis (M. Rosenberg *et al*., 1980). The decrease in absorbance of the lower aqueous phase was used as a measure of cell surface hydrophobicity.

There were contradicting reports with regard to the extent of adhesion observed and the conclusion drawn from the observations. In general, adherence to oil is said to be a specific property of oil-degrading microorganisms (Mel Rosenberg, 1984). Having established this, adherence to hydrocarbons does not necessarily conclude that the microbe has an ability to degrade them because there have been other bacteria reported to have shown adhesion but no degrading capabilities. Since the effect of adhesion is

reported as one of the possible explanations for the hydrocarbon degradation, it was decided to study the effect of adhesion in the bacteria being used in this project.

In order to study the microbial interactions with hydrocarbons it is necessary to wash the extracted cells with PUM buffer. This step is carried out to minimize the effect of the medium that the bacterial cells were cultivated in and to avoid contamination from any other substances that were used in the bacterial isolation. The composition per litre of deionised water is as follows: K_2PO_4, 16.9g; KH_2PO_4, 7.3g; Urea, 1.8g; and MgSO4.7H2O, 0.2g. All the bacterial cultures were grown in duplicates for 48 hours at 25ºC and a higher mixing rate of 180RPM in 10ml of medium with glucose as the carbon source to ensure good growth. After the specified time, all the bacterial cells were harvested by centrifuging at 4500RPM for 90 minutes at 10ºC. The supernatant was discarded and the extracted cells were washed with 4ml of PUM buffer maintained at a pH of 7.1. The cells were washed repeatedly three times to ensure that there are no contaminants left from the culture broth. Once the cells were free from impurities, the cells were re-suspended in the same buffer and optical density measurements were taken at 600nm using a spectrophotometer. After this step the bacterial suspension (8ml) was mixed with 2ml of hexadecane and incubated in room temperature for a further 10 minutes. Following this the mixture is vortexed for further 2 minutes and allowed to settle for 15 minutes. The aqueous phase that is the bottom layer is carefully removed and optical density measured again. This procedure was carried out on *Rhodococcus opacus*, *Rhodococcus ruber* and the control. *Dechloromonas denitrificans* (DSM15892) was used a control in the experiments to ensure that any changes to the optical density measurements can be attributed to the hydrophobicity of the *Rhodococcus* genus. All the bacterial cells were harvested during the exponential phase of growth for the first set of experiments. Another set of experiments were carried with bacterial cells harvested at

various stages of growth in order to ascertain the effect of the growth phase on the phenomenon of bacterial adhesion. This level would give a direct indication to the amount of surfactant produced at the various stages of growth. As normal procedure all the calculations and experimental procedure was carried out in duplicates and the average values were obtained and presented below.

Sample ID	**O.D at 400nm OD_C**	**O.D at 400nm OD_A**	**Hydrophobicity $100(1 - OD_A/OD_C)$**
R. *ruber*	4.12	1.846	55.1941
R. *opacus*	8.114	1.935	76.1523
D. *denitrificans*	5.995	6.37	-6.2552

Table 4.4: Hydrophobicity of cells harvested at exponential phase of growth

In order to verify the hydrophobicity of the bacterial strains the BATH assay was carried out. As discussed previously it can be argued that the adhesion of bacteria to hydrocarbons would not necessarily mean that they are capable of degrading them, but in this case it has been proven that the bacterial strains employed in this study are in fact capable of surviving in the presence of hydrocarbons and also exploit them as their sole carbon source. Hence a positive indication on the BATH assay would mean that bacterial adhesion to hydrocarbons is not only a method of survival but also a method by which the bacteria facilitate hydrocarbon uptake into their cells walls to be utilized as a source of metabolism.

Sample ID	O.D at 400nm OD_C	O.D at 400nm OD_A	Hydrophobicity $100(1 - OD_A/ OD_C)$
R. *ruber* (Lag phase)	0.138	0.127	7.971
R. *ruber* (Exponential phase)	7.635	2.959	61.2442
R. *ruber* (Stationary phase)	8.095	4.146	48.7831
R. *opacus* (Lag phase)	0.163	0.152	6.7484
R. *opacus* (Exponential phase)	9.28	2.014	78.2974
R. *opacus* (Stationary phase)	8.31	2.486	70.0842
D. *denitrificans* (Lag phase)	0.193	0.189	2.0725
D. *denitrificans* (Exponential phase)	6.248	6.017	3.6971
D. *denitrificans* (Stationary phase)	7.484	7.713	-3.0598

Table 4.5: Hydrophobicity of cells harvested at various stages of growth

As we can see in the experimental results presented in Table 4.4 and Table 4.5 it is evident that the bacterial cells of *Rhodococcus opacus* and *Rhodococcus ruber* are extremely hydrophobic in nature. This experiment also further proves that the surfactants produced by each of the bacterial species are fundamentally different in nature. In the case of *Rhodococcus opacus* the higher hydrophobicity further shows that the surfactants are indeed cell bound and even though *Rhodococcus ruber* does have a high affinity to the oil droplets it is not as strong as *R. opacus*. This effect combined with the greater robustness of the *R. opacus* could prove extremely useful when the organisms are grown together or allowed to share a common growth media. Since the

hydrocarbon uptake is complementary to each other, the presence of *R*. *ruber* could indeed enhance the degradability of *R*. *opacus*.

4.3 Surface Tension Measurements and Surfactant Production Levels:

As previously mentioned, one of the principle qualities of the presence of surfactants in the medium is the reduction in the surface tension of water (Ciapina *et al*, 2006). To determine the presence of surfactants in the spent medium, the surface tension was measured for various samples.

The samples were collected aseptically and centrifuged at 4500g/min for 90 minutes at 10ºC to remove the cells. A torsion balance for surface tension and interfacial tension measurements was used (White Electrical Inst. Co. LTD.) alongside a platinum ring having a circumference of 4cms. A total of three readings were taken to minimize errors in calculation. Before any reading was taken, the surface tension of deionised water at room temperature was measured in order to determine the accuracy of the readings obtained. After every reading the platinum ring was sterilized using 75% ethanol and also flame sterilized to remove any cell debris.

In order to determine if the surfactants are cell bound in some of the experiments carried out, two sets of readings were carried out on duplicate samples. In one of the samples prior to centrifugation, the cell culture and broth mixture were vortexed for 2 minutes coupled with sonification for 30 minutes in order to release any cell bound surfactants if any were present and the other was not subjected to any mixing.

Sample ID	Surface Tension Value (dynes/cm)			
	Value 1	**Value 2**	**Value 3**	**Average**
Deionised Distilled Water	71	70.5	71	70.83
2% Glucose 3rd Generation	65.3	55.5	56.0	55.5
2% Glucose 13th Generation	55.0	58	57.5	57.3
Glucose + 3mL Methanol	56.5	58	57.5	57.3
Glucose + 1.5mL Methanol	62	59	59.5	60.1
3% Glycerol at 20ºC	60	60.5	71	60.33
3% Glycerol at 20ºC	59.5	59.5	59.5	59.5
3% Glycerol at 23ºC	64	64.5	64.5	64.33
3% Glycerol at 23ºC	60.5	60.5	61	60.66
3% Glycerol at 25ºC	59.5	60	60.5	60
3% Glycerol at 25ºC	62	60	60.5	60.83

Table 4.6: Surface tension values at different growth conditions of *R .opacus*

The primary requirement of a surfactant is the reduction in the surface tension value of the growth media. This gives a strong indication of the potency of the surfactant being produced. Any surfactant that is capable of reducing the surface tension of water by more than 20 dynes/cm is considered a good candidate for further study. The surface tension values obtained in the experiments using *Rhodococcus opacus* and *Rhodococcus ruber* are very promising. In the initial experiments carried out with hydrophilic carbon sources as presented in Table 4.6 above it was noted that the surface tension values were not considerably reduced. The reason for such behaviour is primarily due to reasons of why surfactants are produced in the first place. The need for surfactant production by an organism arises from the fact that the carbon source is inaccessible by the organism. But since this was not the case when hydrophilic carbon sources were utilized, surfactant production was not required but when grown on alkanes a high degree of surfactant production can be reported.

This evident from Table 4.7 where it can be seen that the average surface tension values were significantly lower with the use of a hydrophobic carbon source such as n-hexadecane.

Sample ID	Surface Tension Value (dynes/cm)			
	Value 1	**Value 2**	**Value 3**	**Average**
Deionised Distilled Water	71	72	71	71.33
$(NH_4)_2SO_4$ T = 6 Hours	63	64	64.5	63.83
NH_4NO_3 T = 6 Hours	61	60	61	60.66
$NaNO_3$ T = 6 Hours	66	66.5	67	66.5
KNO_3 T = 6 Hours	68	67	67.5	67.5
$(NH_4)_2SO_4$ T = 29 Hours	62	62.5	60	61.5
NH_4NO_3 T = 29 Hours	68	67	67	67.33
$NaNO_3$ T = 29 Hours	58	56	56.5	56.83
KNO_3 T = 29 Hours	60	65	60	61.66
$(NH_4)_2SO_4$ T = 52 Hours	55	57	54.5	55.5
NH_4NO_3 T = 52 Hours	56.5	54	57	55.8
$NaNO_3$ T = 52 Hours	43	39	39.5	40.5
KNO_3 T = 52 Hours	48	45	46.5	46.5
$(NH_4)_2SO_4$ T = 100 Hours	54	54.5	53.5	54
NH_4NO_3 T = 100 Hours	56	55.5	57	56.16
$NaNO_3$ T = 100 Hours	31	32	29.5	30.83
KNO_3 T = 100 Hours	47	44	46.5	45.8

Table 4.7: Surface tension values using various nitrogen sources and n-hexadecane for *R. ruber*

In order to quantify the surfactants produced which resulted in the drop in surface tension, we used a method first devised by Du Bois *et al* that makes use of phenol and sulphuric acid. This method was developed in order the quantify sugars in a given sample, and is very simple to carry out. The main principle of the reaction involves using the heat of the reaction to drive the formation of the absorbing compound and can be carried out with only a few chemicals and a spectrophotometer.

One of the major disadvantages of this method is its variable absorbance response to different sugars and this makes the assay unpredictable and difficult to use on biological samples (Taylor, 1994). The original assay developed by Du Bois *et al* was further modified by Rao & Pattabiraman, which gave slightly better absorbance. Taylor (1994) made further modifications to this assay that finally was able to reduce the variability of the response even further. The R-P assay was modified by increasing the acid content to approximately 80% and additionally by using stricter temperature control the variability of response to the various types of sugars was further reduced.

This assay was specifically chosen in this project since it was hypothesized and proven that the genus *Rhodococcus* is known to produce surfactants which were glycolipids in nature composed of trehalose mono- and dicorynomycolates (Karpenko *et al*., 2006, Ciapina *et al*., 2006; Mutalik *et al*., 2008).

In this project, the Taylor method was used since it was proven to yield more accurate results and has been used in similar studies (Ciapina *et al*., 2006). The entire operation was carried out in a fume hood since concentrated phenol and sulphuric acid

were employed in this assay. Before the experiment was started two things needed to be done:

1. Stock solution of 90% concentration of phenol was prepared.
2. Glucose solutions of known concentrations-20μg, 40μg, 60μg, 80μg, 100μg are prepared. The above experiment is first carried out using the above prepared standards and the standard curve is thus prepared.

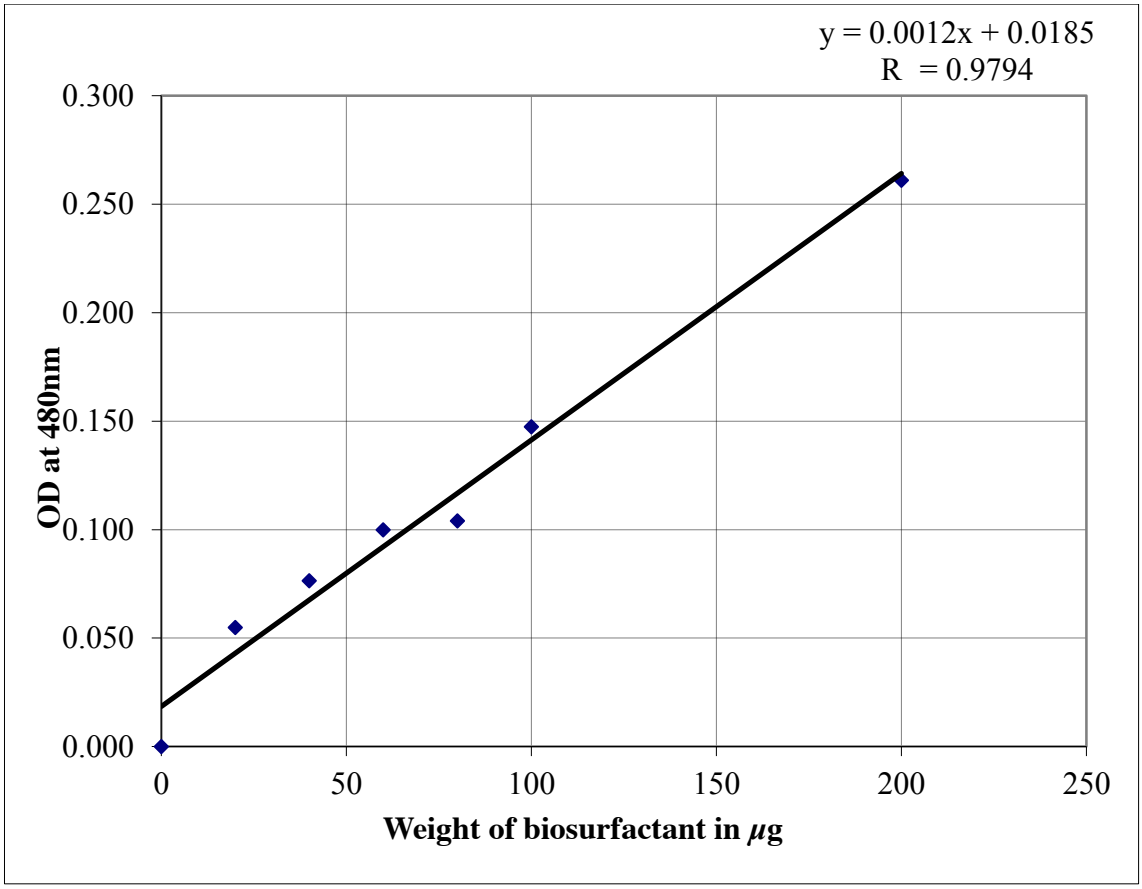


Figure 4.1: Glucose standard curve for Phenol-Sulphuric Acid Assay

The optical density values that were obtained during the assay are compared to the glucose standard curve Figure 4.1 to obtain the relevant glucose concentration, which would correspond directly to the surfactant concentration.

At first 1.0mL of the sample was taken in clear dry test tube. The bacterial sample, which was used as the sample prior to carrying out the experiment, was subjected to sonification (Fisher Scientific, UK) for 30 minutes and then centrifuged at 4500g for 60 minutes. The sonification is required to release the cell bound supernatants (Ciapina *et al*., 2006) and the centrifugation allows for the bacterial cells to be separated so that they do not affect the specificity of the assay. After the first step 3mL of concentrated sulphuric acid was added quickly along the sides of the test tube and vortexed for two minutes. The reaction was allowed to proceed for one minute. The test tubes were then cooled to room temperature using a water bath to reduce the temperature quickly. To this 50μL of 90% phenol was added and the reaction was allowed to proceed for at least 30 minutes. The absorbance was taken using a spectrophotometer at 480nm. For this experiment we have used water, sulphuric acid and 90% phenol without the sugar as a blank.

The above procedure was carried out to each of the samples that were obtained during the course of the growth curve. The absorbance values are compared to the standard curve prepared using the glucose standards, to quantify the amount of glycolypid class surfactants being produced. The concentration of glucose is directly reported as the concentration of the surfactant.

The below figures (Figure 4.2, Figure 4.3, Figure 4.3) represent the data obtained for surfactant concentrations obtained when *Rhodococcus opacus* and *Rhodococcus ruber* were grown on various nitrogen sources. The these experiments both the bacterial strains were grown on n-hexadecane as the sole carbon source to aid in biosurfactant production.

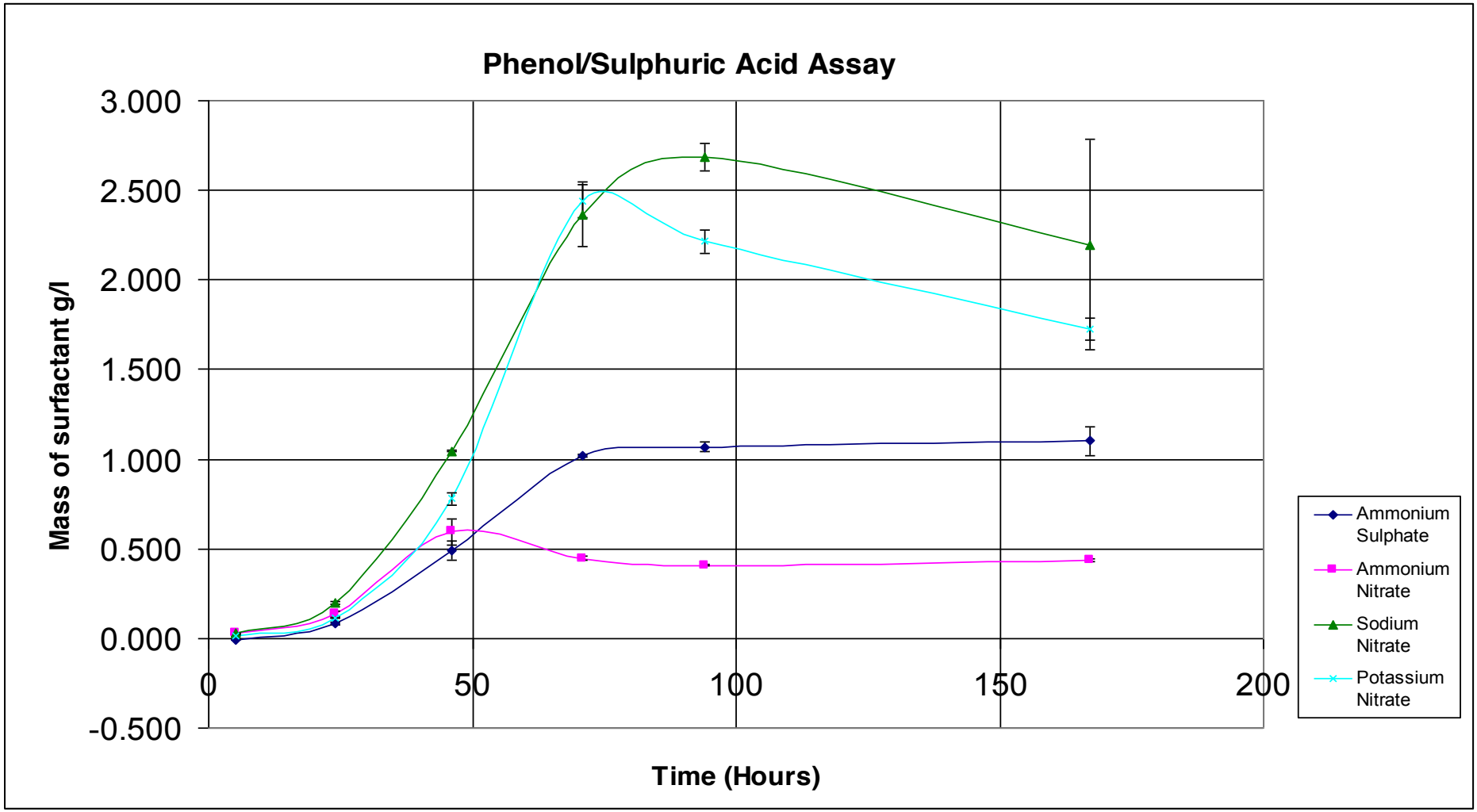


Figure 4.2: Surfactant concentrations of R. *opacus* grown on various nitrogen sources

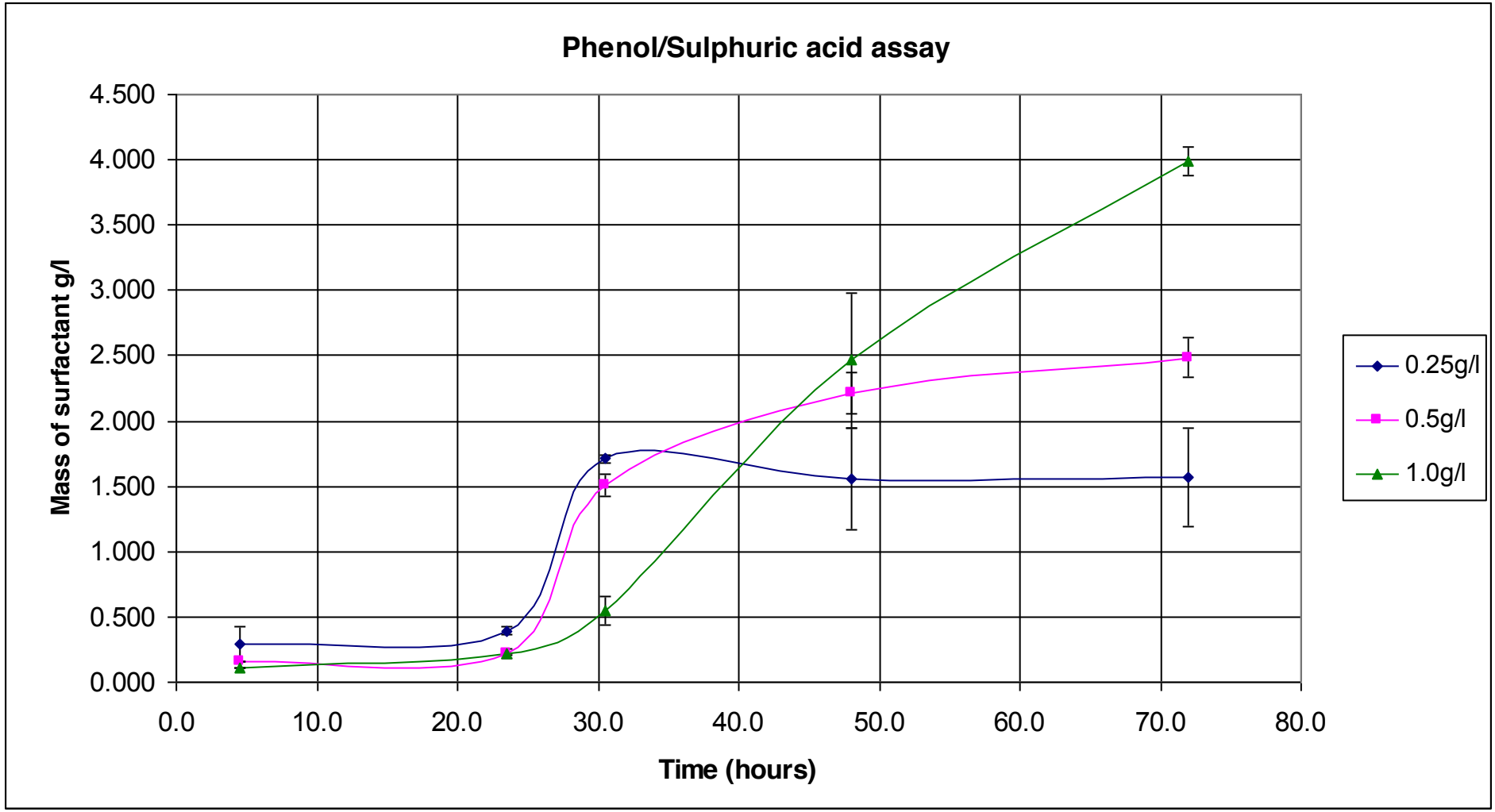


Figure 4.3: Surfactant concentrations of R. *opacus* grown on different concentrations of sodium nitrate

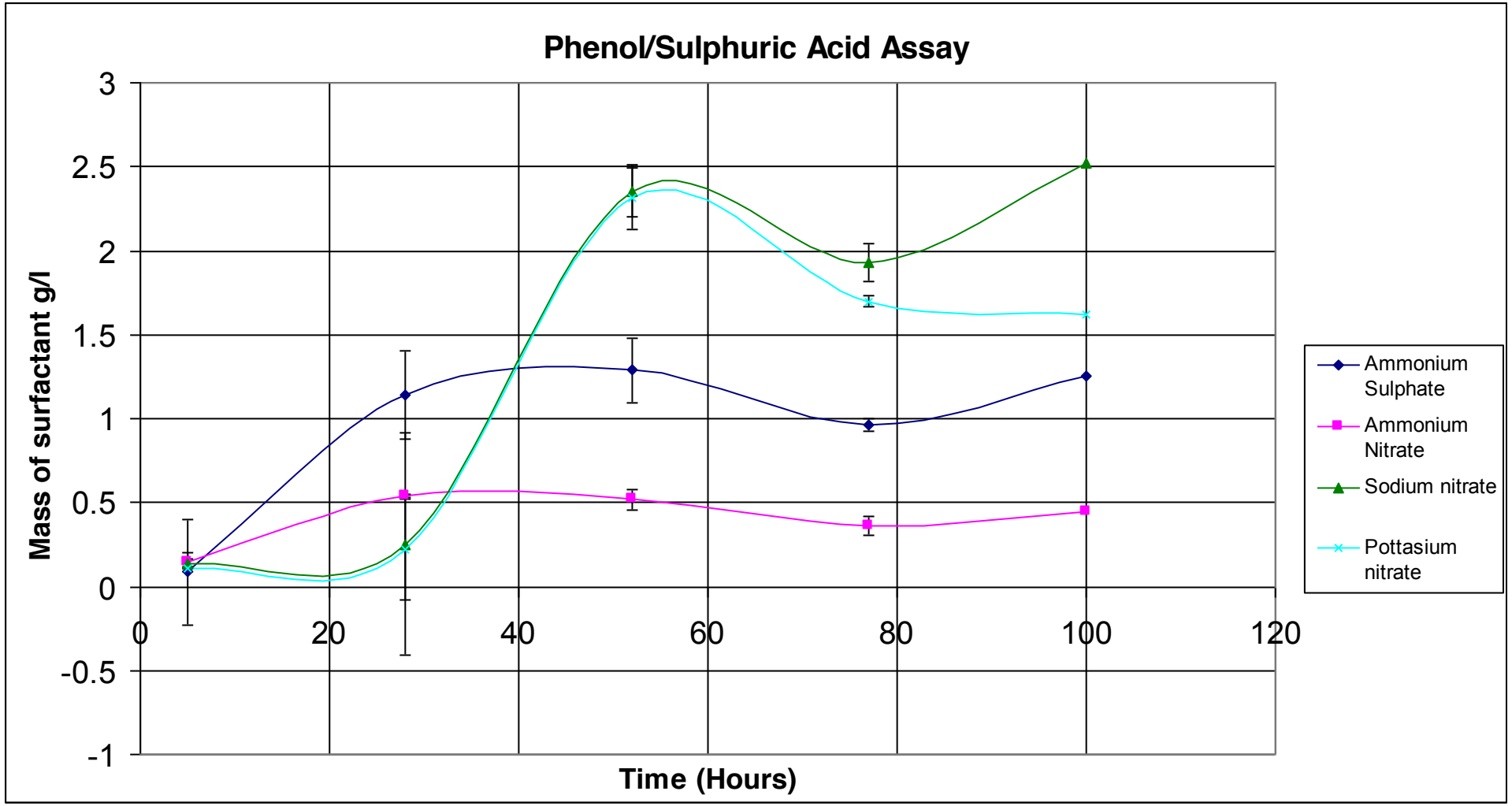


Figure 4.4: Surfactant concentrations of R. *ruber* grown on different nitrogen sources

The modified R-P assay was used to quantify the surfactants that are being produced by the bacterial strains. As seen in the previous chapter, the data represented in Figure 4.2 and Figure 4.4 show that medium optimisation seems to be the key factor that determines the surfactant concentration. The assay was carried out on samples that were collected during the optimisation experiments carried out using various nitrogen sources. The graphs indicate that the surfactant levels are almost three times higher in the presence of sodium nitrate and potassium nitrate than in the presence of ammonium salts. The lower levels are production could be explained by the fact that surfactant production is growth related, the presence of ammonium salts are not favourable for bacterial growth and hence the lower surfactant levels. On the whole the surfactant concentrations are considered moderate. Increased concentration of nitrogen source is seen to improve the production levels and indicates that higher production levels could be achieved with the optimum use of growth media and conditions.

4.4 Surfactant Extraction Methods:

For the purposes of this work a high degree of surfactant purity was not required. This is due to the requirement that eventually the bacterial cultures are to be cultivated together in a reactor and in such a scenario, the mere presence of the surfactant in the medium would be sufficient to reduce the surface tension. All the extraction that has been carried out in this work is primarily to test the presence of surfactants and their general efficacy. Hence surfactant extraction was primarily carried out using solvent extraction, one of the many methods employed in obtaining crude extracts (Sen *et al*., 2006). This method was chosen due to the ease of setting up the experiment that would suit better due to the high volume of samples that were analysed. Another reason to choose solvent extraction was because of the minimal set-up that was

required. The protocol used to extract the surfactants was modified to suit the scale of the operation.

In order to obtain crude surfactant, 50 ml of the culture medium was sonified for 15 minutes to extract any cell bound surfactants along with any present in the culture medium. To this 50 ml of chloroform and 100 ml of methanol were added and blended using a vortex for 2 minutes. The entire operation was carried out inside a fume hood due to the nature of solvents that were employed in this method. After the first step another 50 ml of chloroform was added and the entire mixture was vortexed for a further 30 seconds. In order to create a partition of the solvents 50 ml of deionised distilled water was added to vortexed mixture and blended for another 30 seconds. The entire contents were then filtered into a 500 ml graduated cylinder using a Whatman membrane of 0.2 μm pore diameter and Hartley funnel setup. To aid in the filtration a vacuum pump was attached to the Hartley funnel. The solvent mixture was allowed to settle for 10 minutes before the top alcoholic layer was carefully removed by suction and to be certain of complete removal a small layer of the chloroform layer was removed as well.

The extracted layer is expected to contain any cell bound and free surfactants that were present in the culture medium. In order the make sure that bacterial cells have not passed through the Whatman filter paper, the chloroform layer was sub sampled and a streak plate prepared using Medium B and agar was carried out. The rest of the extraction procedure is carried out using a Buchi Rotavapor R114 coupled with a Buchi water bath B480 fitted inside a fume cupboard. The choice of chloroform in this solvent extraction procedure is primarily due to the low boiling point of 61.2 ºC that makes the use of a rotavapor convenient. Prior to the experiment dry beakers are placed in an oven

and the dry weight measured using an analytical balance. The residue from the rotavapor are collected in the pre-weighed glass beakers and placed in an oven to remove any residual chloroform and placed a desiccator over-night to remove residual water. The crude extract is ready to be analysed for surfactant activity.

Sample ID	**Surface Tension Values (dynes/cm)**			
	Value 1	**Value 2**	**Value 3**	**Average**
2.5 g/L crude surfactant in Medium B	43	39	39.5	40.5
5 g/L crude surfactant in Medium B	39	35.5	31	35.2
7.5g/L crude surfactant in Medium B	34.5	32	31.5	32.7
10.0g/L crude surfactant in Medium B	31	32	29.5	30.8
12.5g/L crude surfactant in Medium B	31.5	31.5	30.5	31.2

Table 4.8: Surface tension values obtained using crude surfactant obtained by solvent extraction and then dissolved in Medium B for *R. Ruber*

From the data obtained, it is very clear that the surfactant production is growth associated. The surfactant quantification and drop in surface tension values are reasons for this conclusion. Once crude extracts of the surfactants were obtained using solvent extraction, it was possible to further test the effectiveness of the surfactants produced. A low surface tension value of 30 dynes/cm was achieved using the crude extract. This low value indicates that the surfactant is very effective in lowering surface tension.

Chapter 5: Mixed Culture Reactor Design and Operation

This chapter describes in detail the operation of mixed culture reactors. Two different approaches have been investigated in this study. In one method two strains of bacteria namely *Rhodococcus opacus* and *Rhodococcus ruber* have been grown together in a conventional 3L automated batch reactor vessel. The other method involved the use of a specially constructed membrane reactor, in which by the use of membrane partitions the two strains of bacteria are physically separated. In order to assess the behaviour of the two bacterial strains grown together, smaller scale flask culture studies have been conducted prior to scale up and compared to the results obtained from the 3L automated batch reactor. The various advantages and disadvantages of using mixed culture systems have been discussed previously in Chapter 1.

5.1.1 Membrane Selection and Preparation:

The initial studies were carried out on a membrane filtration device, Pellicon XL filter module (Millipore, UK). Different membrane materials such as polyethersulfone, nylon nets and PVDF were considered before choosing the Pellicon XL microfiltration cassettes for the initial membrane studies. These were constructed using hydrophilic polyvinylidene fluoride (Durapore membrane), which were chosen to be fitted on the membrane reactor. The PVDF membranes are well known for the low protein binding and high product recovery. A hydrophilic polypropylene PVDF membrane was chosen to avoid build up of n-hexadecane during the filtration process. The small pore diameter of 0.22 μm ensures that there would be no exchange of bacterial cells but would allow for the transfer of surfactants and culture media through the filtration device.

The construction of the Pellicon XL microfiltration cassettes were ideal due to the way they replicate in smaller scale the actual functioning of the membrane reactor. Exactly like the membrane reactor they have two streams in cross flow separated by a Durapore PVDF membrane. The construction also allowed for repeated use of the membrane after a cleaning cycle using 0.1% Tween 80 solution and the sterilisation of the membrane module was carried out using 0.05% sodium azide solution. The module was stored at 4ºC with 10 mL of sodium azide solution injected into the module to prevent any bacterial growth during storage.

5.1.2 Filtration Trials:

Prior to every run the filter module was connected to the centrifugal pump using the tubing supplied and the flow rate was adjusted to 33 mL/min. This was due to the construction of the cassettes, which need to be operated under certain tangential flow rates in order to maintain the integrity of the device. In order to flush out the storage solution, deionised distilled water was pumped through the membrane until 250 mL was collected as permeate. Following this the membrane was conditioned with Medium B until a further 250 mL was collected as permeate.

In order to determine the efficiency of the chosen membrane there were primarily two requirements. It was necessary that the surfactants pass through the membranes and that the bacterial cells do not. Hence the membrane trials were carried out in two different ways. In one of the experiments, the culture broth was sonified for 30 minutes in order to separate any cell bound surfactants and then centrifuged at 4500 RPM for 1 hour in order to separate the cells from the culture broth. The other experiment was carried out using the culture medium without centrifugation. There is always a possibility of permeate contamination in the presence of cell debris hence the

requirement of the first experiment. This would reduce the risk of contamination and help determine the filtration capacity of the chosen membrane. In both the experiments the permeate was collected and stored at -30ºC to analyse for surfactant concentration using the Phenol-Sulphuric acid assay as described in Chapter 4 and also plated on agar plates to observe bacterial growth.

Table 5.1 and 5.2 below are used to demonstrate that the membrane present inside the Pellicon XL filter module is successful in allowing the surfactants to pass through which is evident from the surfactant concentration measured after being filtered through the device. The surfactant concentration after filtration is measured using permeate that is collected and stored at -30ºC.

Sample ID	**Optical Density at 600 nm**	**Surfactant Concentration g/L**
Blank A	0.012	-0.005
Blank B	0.038	0.016
Pre-Filtration	0.533	0.429
Pre-Filtration	0.526	0.423
Filtered A	0.230	0.176
Filtered B	0.295	0.230

Table 5.1: Membrane test using *Rhodococcus opacus*

Sample ID	**Optical Density at 600 nm**	**Surfactant Concentration g/L**
Blank A	0.010	-0.007
Blank B	-0.009	-0.023
Pre-Filtration	1.120	0.918
Pre-Filtration	1.121	0.919
Filtered A	0.408	0.325

Filtered B	0.538	0.433

Table 5.2: Membrane test using *Rhodococcus ruber*

The choice of the Pellicon XL filtration system was primarily due to the incorporation of the Durapore membrane filters. Comparison of various membranes specifications showed that the hydrophilic Durapore membranes bind far less protein than other commonly used membrane materials such as nylon, nitrocellulose or PTFE. Another important factor was the ability to withstand sterilisation by autoclaving to temperature up to 121ºC. The hydrophilic nature of this membrane was suited to the nature of the experiments conducted where n-hexadecane was used as the sole carbon source and hence this membrane would prevent accumulation of hexadecane on the membrane surfaces. The results obtained from using the Pellicon XL device showed that after filtration only around half of the surfactants were passing through the membrane. This can be attributed to the cassette form factor of the device, which did not allow a very high inlet pressure due to the closed construction of the device. Also a minimum working volume of 15 mL was required with a residual volume of 3.2 mL that did not allow for complete removal of filtrate from inside the device. In spite of these limitations the primary goal of the experiments were achieved. The results show that the surfactants are able to pass through the membrane. Permeate obtained from the experiments were inoculated on solid agar plates that showed no sign of bacterial growth indicating that the bacterial cells were not passing through the membrane. Hence this membrane was chosen for further studies using the membrane reactor.

5.2 Mixed Culture Experiments Using Flask Cultures:

Two sets of experiments were carried out to observe the effect of bacterial co-cultures. In one of the experiments, crude surfactant extract from *Rhodococcus ruber* was obtained and added along with the culture media prior to autoclaving and

inoculated with *Rhodococcus opacus*. In order to obtain a crude extract, solvent extraction using chloroform and methanol was used (Sen *et al*., 2006) and modified to suit this work. Chloroform and methanol in 1:2 ratio was added to culture medium after sonification to release the cell bound surfactants. The solvent mixture is then partitioned and the chloroform layer along with the surfactants is extracted. This is further purified using a Buchi Rotavapour coupled with a water bath (procedure detailed in Chapter 4). The second set of experiment consisted of growing the individual bacterial strains, and different concentrations of the two strains together.

The first experiment involved the growth of *Rhodococcus opacus* in the presence of crude surfactant extracted from *R. ruber* at two concentrations (1g/L and 3g/L). This was carried out to ascertain the effect of the presence of surfactant from another bacterial strain as this would be similar to the growth condition prevalent in the membrane reactor discussed in this chapter. The media was prepared together with n-hexadecane and accurately weighed quantities of the crude surfactant were added to the culture media prior to autoclaving at 121ºC for 15 minutes. Un-inoculated medium B was used as the control and all flasks were grown in duplicates. The flasks were inoculated with 1 mL of seed culture harvested during exponential phase of growth. The temperature was maintained at 25ºC and mixing was provided using Stuart orbital incubator 5150 (UK) that was set at 170 RPM.

The second experiment involved the growth of both the bacterial strains individually and together by varying the concentration of each stain. This was carried out to study the influence of each bacterial strain on the other and compare growth data to those flasks that were inoculated with only one strain each. In one set of duplicate

flasks the seed inoculum consisted of 25 % *R. ruber* and 75% *R. opacus* and 75 % *R. ruber* and 25% *R. opacus* in the other set of duplicate flasks. The growth parameters were maintained the same as the previous experiment.

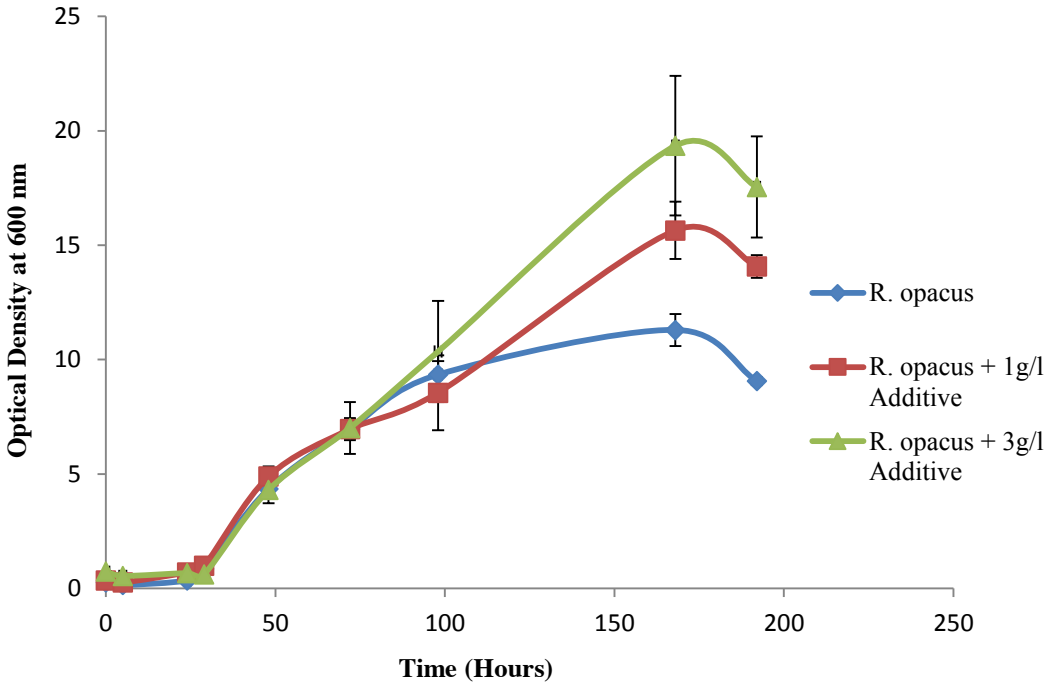


Figure 5.1: *R. opacus* grown with *R. ruber* surfactant extract at different concentrations

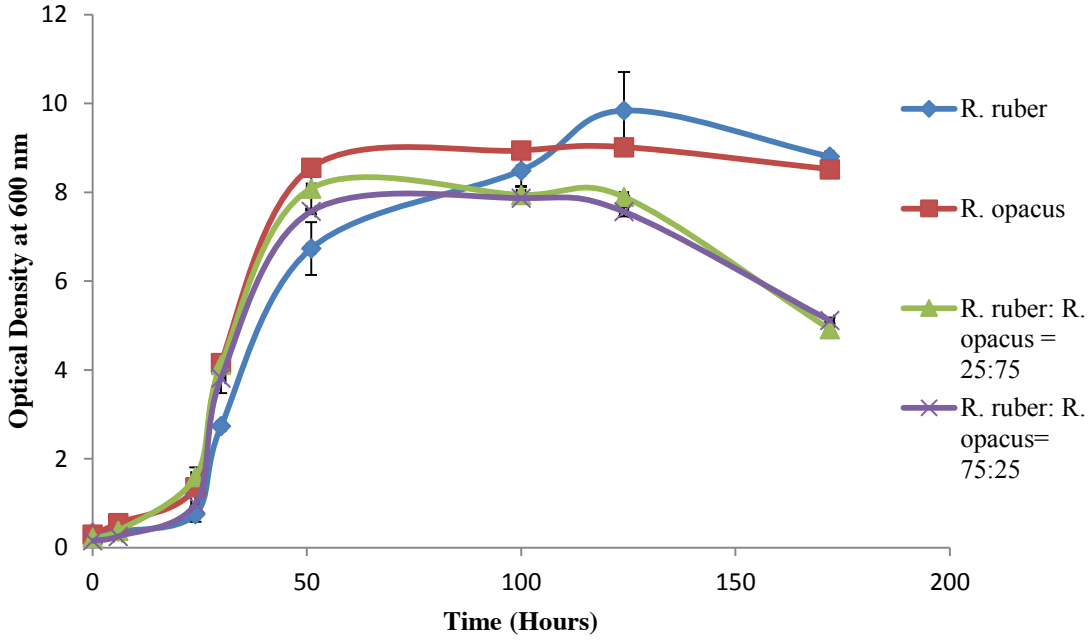


Figure 5.2: Bacterial strains grown together at different concentrations

In all the experiments carried out so far as described in the previous chapters, the growth of the bacteria was observed when grown separately. Hence before the scale up was done it was required to observe the growth of bacterial cells when cultivated together. Crude surfactant obtained by surfactant extraction using solvents as described in Chapter 4 was stored at -30ºC to prevent contamination. Figure 5.1 represents data obtained from experiment one in which *Rhodococcus opacus* was grown in the presence of crude surfactant extract obtained from *Rhodococcus ruber*. Because the extract was not lyophilised, accurately weighed quantities of the extract were quantitatively transferred to the culture media prior to autoclaving. This was carried out to elicit the same conditions that would be prevalent when the two bacterial strains were grown in the membrane reactor. The fact that the cell culture was able to grow in the presence of externally produced surfactants is very important as this implies that the membrane reactor used this project would work. The concept of physically separating the cells to avoid direct interaction but allow the interaction of any secondary metabolites to be produced could potentially be exploited on a larger scale.

The second experiment illustrated by Figure 5.2 above, involved the growth of *Rhodococcus ruber* and *Rhodococcus opacus* together to assess the effect of the physical interaction between the two bacterial strains. From the growth data obtained in this experiment, it can be seen that the growth of the individual culture of *R. ruber* and *R. opacus* stronger than when the strains were grown together at different concentrations. The varying percentages employed for this particular experiment was chosen to simulate the growth conditions in a mixed-culture reactor system where the rate of growth of the individual strains are not necessarily equal to each other. The results obtained in this experiment clearly indicate that when the bacterial strains are cultivated together they have a detrimental antagonistic effect on each other, which is evident from the growth curves. This validates the work carried out using the membrane

reactor where this scenario would be avoided by physically separating the bacterial cells from each other. The use of a membrane reactor would thus negate any antagonistic effect of the bacterial strains towards each other but because the exchange of culture media is permitted it would be possible to exploit the individual capabilities of chosen bacterial strains.

5.3.1 Mixed Culture Experiments with Automated Batch Reactor:

The mixed-culture studies consisting of *R. opacus* and *R. ruber* were carried out in a 3L fermentation vessel made by Applikon Biotechnology. The BioBundle fermentation system used in these experiments consist of one ADI 1010 Bio Controller, one ADI 1025 Bio Console and one 3L jacketed autoclavable glass vessel that is attached to a high precision motor, pH, dissolved oxygen and temperature probes as presented below in Figure 5.3.

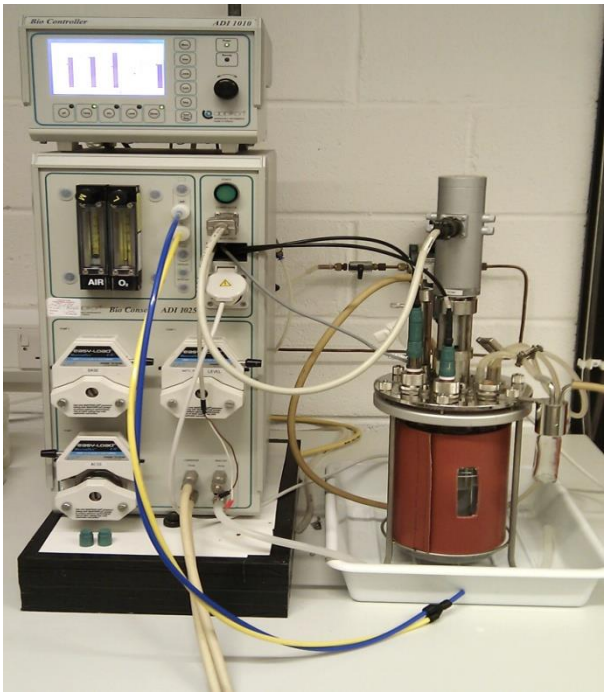


Figure 5.3: BioBundle Fermentation system

All the operating parameters in this reactor system (pH, temperature, dissolved oxygen level and stirrer speed) can be controlled manually in real time or automated to run unsupervised using the ADI 1010 Bio Controller that was connected to a computer using the BioBundle software.

5.3.2 Preparation and Operating parameters:

Three sets of experiments were carried out using the batch reactor, one experiment each with the individual bacterial strains *R. opacus* and *R. ruber* and another experiment using both the bacterial strains at equal concentrations. Prior to every experiment the bacterial cells were sub-cultured and taken through three generations in the chosen carbon source (n-hexadecane) before inoculation was carried out to ensure that there was no contamination in the seed inoculum. All the experiments were carried out with a working volume of 1.5 L Medium B and 15 mL of n-hexadecane as carbon source. In order to avoid contamination, the entire glass vessel along with the culture medium and the tubing were autoclaved at 121ºC for 15 minutes prior to inoculation. The pH and dissolved oxygen probe were sanitized using methylated alcohol solution. The air vent in the reactor vessel was attached with 0.22 μm air filter in order to prevent outside contamination and the extraction vent was sterilized using alcohol solution. Prior to every run the inoculation septum on the batch vessel was inspected and changed if required.

The operating parameters were kept constant throughout the course of the three experiments in order to be able to compare the results. Prior to inoculation, the pH probe was calibrated and the pH of the culture medium was adjusted to 9 using 2M NaOH solution. The dissolved oxygen probe was calibrated with air at 100% before being attached to the batch vessel and the rotor speed was set to 500 RPM. The

BioController was used in these experiments to monitor the pH, dissolved oxygen and control the rotor speed and temperature. After the pH of the medium was initially set to 9, the BioController was not used to auto-correct and maintain the pH since it was required to assess the fluctuation in the pH of the culture medium. The temperature of the culture medium was maintained at 25 ºC using an external jacket that was attached around the body of the reactor vessel after all the probes and motor were attached to the reactor vessel. Inoculation was carried out using 15 mL of cell culture harvested during the exponential phase of growth. After inoculation was carried out samples of the culture medium were aseptically withdrawn at regular intervals to measure the optical density and surface tension values.

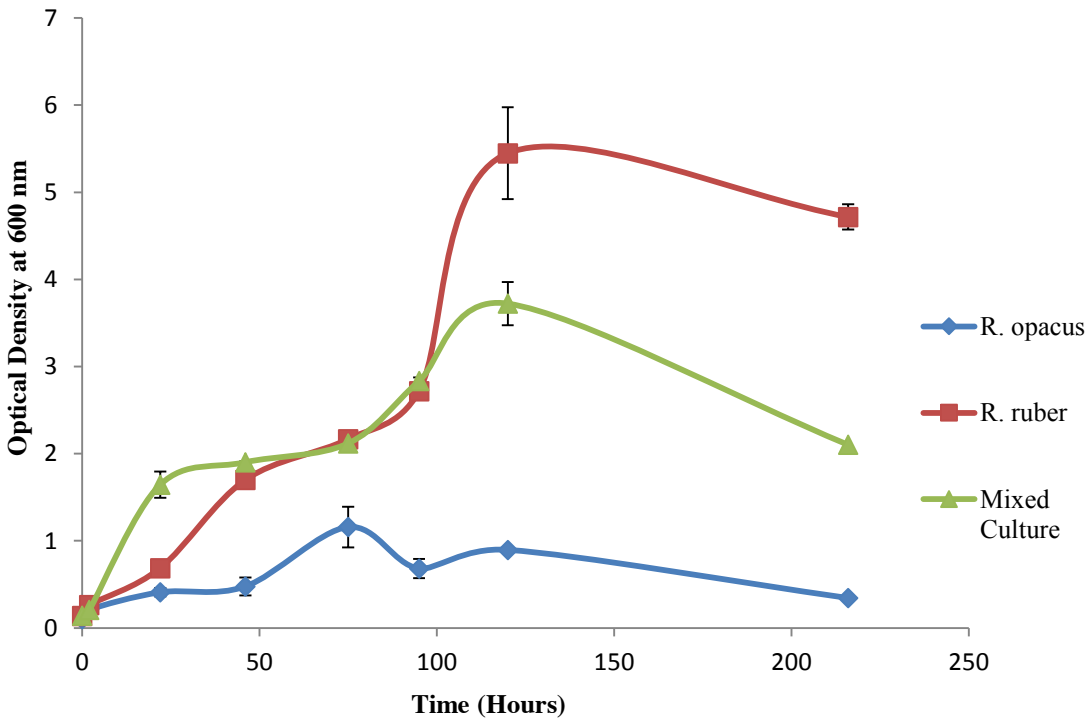


Figure 5.4: Growth of individual and mixed bacterial cultures

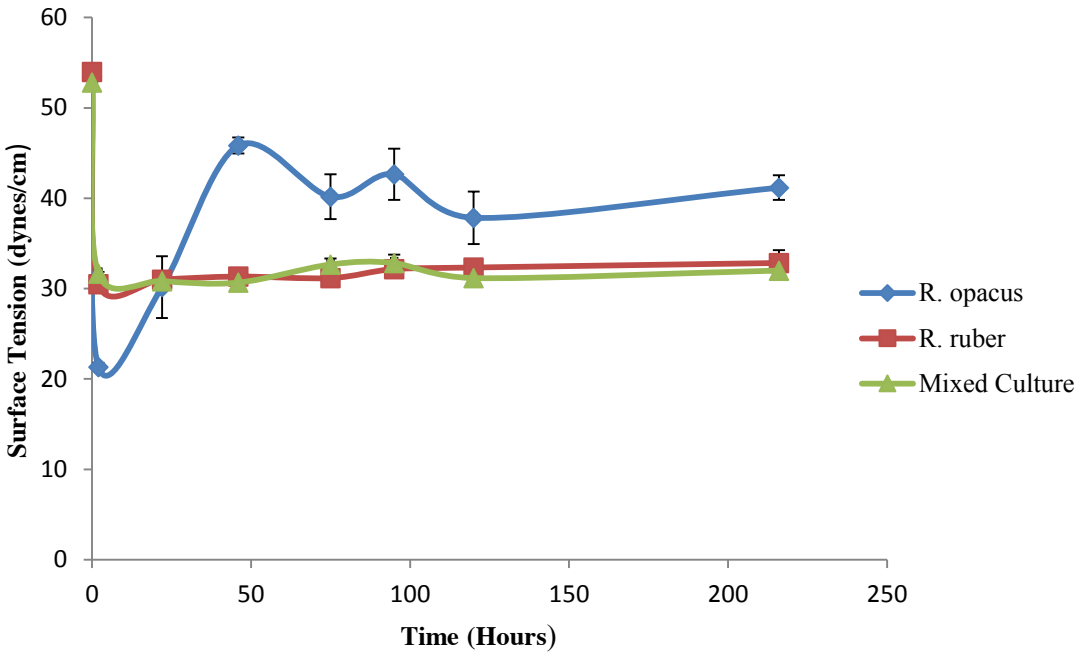


Figure 5.5: Surface tension of culture media during bacterial growth

This result can be compared to the automated batch reactor where a similar pattern can be observed. The growth in the automated batch reactor for *R. opacus* and *R. ruber* was not as good as the smaller scale flask cultures. This could be due to the scale up conditions not being entirely favourable for growth but was mainly carried out to assess the difference in growth and surfactant production on a larger scale. In both the cases, it can be inferred that the presence of *R. ruber* either directly or indirectly has an influence on both the total growth and capability to reduce the surface tension of the growth media considerably. This is further proved in the experiment with *R. opacus* using surfactant additives obtained from *R. ruber*. This experiment clearly shows that the presence of surfactants has an overall positive effect on the growth of R. *opacus* in the presence of a hydrophobic carbon source where the only method of carbon uptake in this bacterial strain is assimilation by production of surface tension reducing agents. It is also possible to conclude by comparing these two results that the enhanced growth of R. *opacus* is not due to the physical presence of R. *ruber* but the presence of surfactant activity induced by R. *ruber* in the culture media.

5.4 Mixed Culture Experiments with Membrane Reactor:

A Durapore membrane filter (Millipore, UK) was used in the membrane reactor. The same polypropylene hydrophilic membrane with pore diameter of 0.22μm and membrane diameter of 47 mm was used, as was tested with the Pellicon XL filter module. The design and functioning is very similar to the Pellicon XL cassettes that were initially used in the membrane trials. Four specially constructed filter modules were used in this experiment, each being separated from each other using the Durapore membrane. The design of the membrane reactor was carried out as part of an undergraduate project and was fabricated in the engineering facility within the department of Engineering and Physical Sciences at Heriot-Watt University. In order to prevent leakages in between the chambers, a rubberized seal was placed between the plates holding the 0.22μm membranes and outside coated with natural petroleum jelly to seal any gaps present in the setup.

Additional membrane modules were constructed and due to the simplicity of the experimental arrangement, membrane modules could be added in pairs to increase the working volume if required. In comparison to the cassette filtration unit, the operating pressure of the screen filters is lower due to the way they are used in this experiment. However this does not affect this work because only lower pumping speeds were utilized in order to maximize the retention time of the bacteria inside the membrane modules. This is crucial since this is the underlying principle behind the working of such a reactor. Since a cross flow is maintained and each bacterial strain is contained in different flow streams, the only exchange that takes place i.e. culture medium and metabolites happens through the membrane present between the interconnected filter modules. This principle is unique to this type of reactor and potentially could be used with any two bacteria or co-cultures.

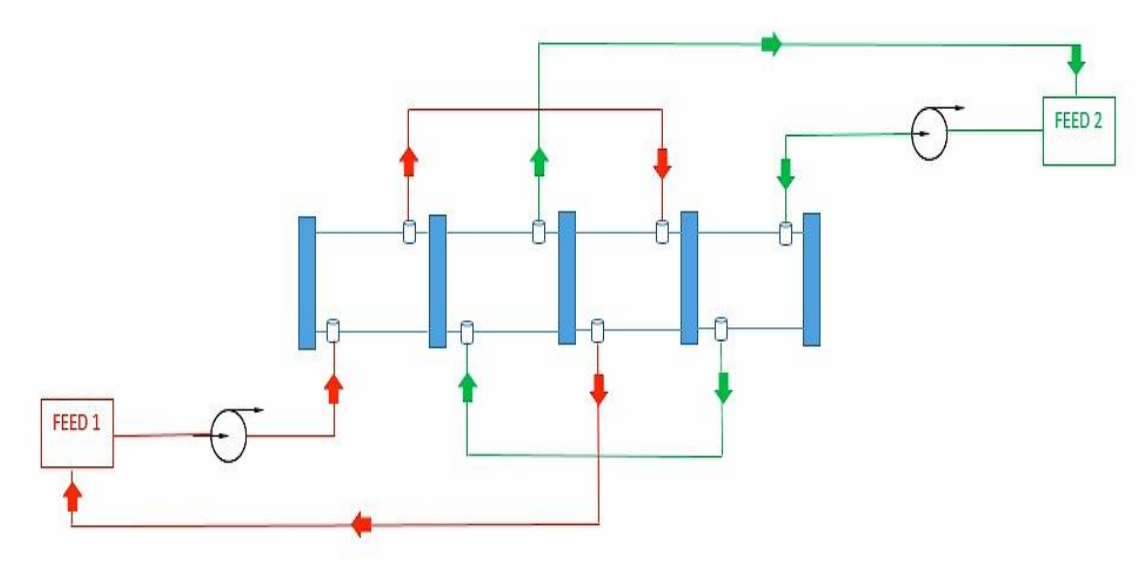


Figure 5.6: Membrane reactor flow diagram

As illustrated above in Figure 5.6, each filter module has two inlets through which they are connected to the other filter modules. The sterilized flexible tubing is arranged in such a way so as to allow for maximum flow area. A cross flow mechanism was followed throughout all the experiments using the membrane reactor.

For all the experiments the filter modules were connected to allow for cross flow of culture medium between every alternate module. This was to ensure the maximum exchange of media and metabolites between the individual filter modules. The media feed for the two opposing streams (Stream 1 and Stream 2) was maintained at a constant temperature of 25ºC using a water bath and stirring was provided by using magnetic stirrers placed within the feed containers. Centrifugal pumps were attached to each of the feed containers and set at 50 RPM to provide a steady flow of media through the filter modules. All the media along with the carbon source, the membrane reactor completely setup as shown in the Figure 5.7 below and the tubing were sealed and autoclaved at 121ºC for 15 minutes except when the carbon source was glucose when the sterilisation was carried out at 110ºC for 10 minutes to avoid degradation of glucose.

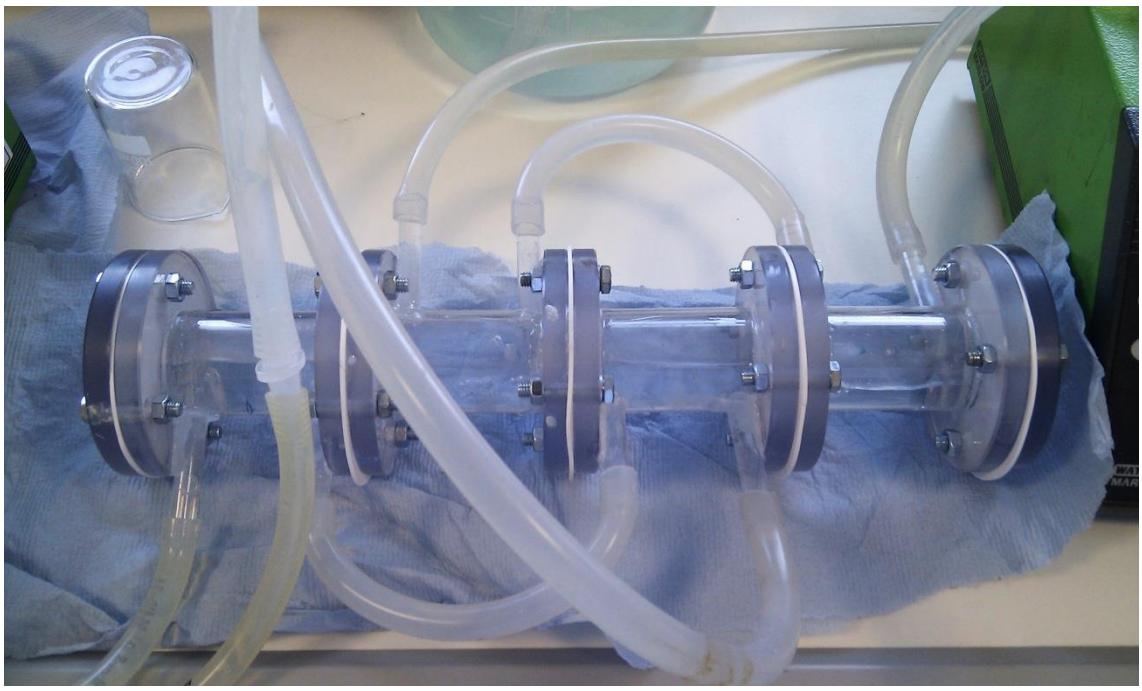


Figure 5.7: Initial setup of the membrane reactor and tubing to allow cross flow

Before the experiments with live bacterial cultures were conducted, the membrane reactor was autoclaved at 121ºC for 15 minutes and run using only water on both the feeds. This was carried out to verify that the autoclaving process did not damage the membrane or the glue used to attach the filter modules and to ensure that there was no leakage from the membranes. Another aspect of the membrane reactor that needed to be verified was if the bacterial cells were capable of passing through the membrane separating the filter modules and if there was media exchange between the modules.

The first experiment to be conducted on the membrane reactor was carried out by using *Rhodococcus ruber* grown on glucose in stream 1 and sterile deionised water was used in stream 2. Figure 5.8 below illustrates the flow regime, where black arrows represent Stream 1 containing *Rhodococcus ruber* and red arrows represent Stream 2, which contained sterile deionised water. The feed 1 containing the bacterial cells was

pH adjusted to 9 using 2M NaOH solution and feed 2 was not pH adjusted. During the course of the experiment samples were obtained from the feed containers at regular intervals to measure optical density and pH. Once the experiment had proceeded for a few days a sample was obtained from stream 2 containing sterile deionised water and plated on agar in order to determine if bacterial cells were passing through the membranes. From visual observation it was evident that the bacterial cells were not passing through the membrane as there was no growth observed where the sample was inoculated in a plain un-inoculated media that was used to culture the bacterial strains as seen in Figure 5.10.

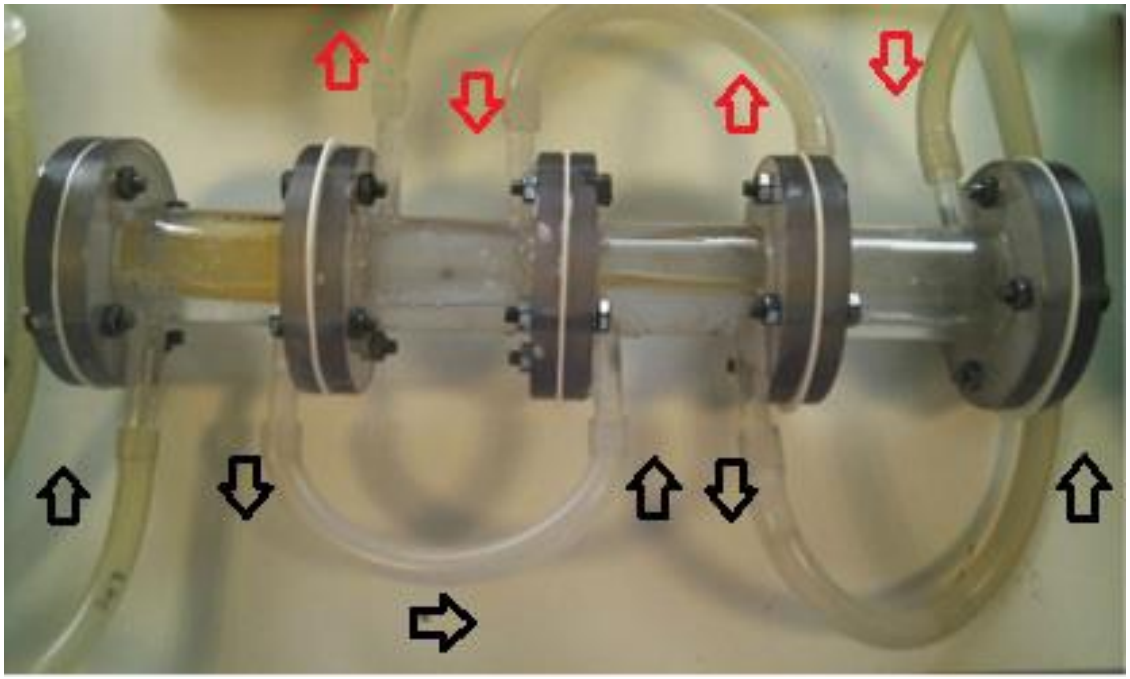


Figure 5.8: Membrane reactor with R. *ruber* in stream 1 and DI water in stream 2

The remaining experiments were carried out with *Rhodococcus ruber* in stream 1 and *Rhodococcus opacus* in stream 2. As with the previous experiment the feed container was inoculated with 15 mL of the seed culture that was harvested during the exponential phase of growth after being sub-cultured through three generations.

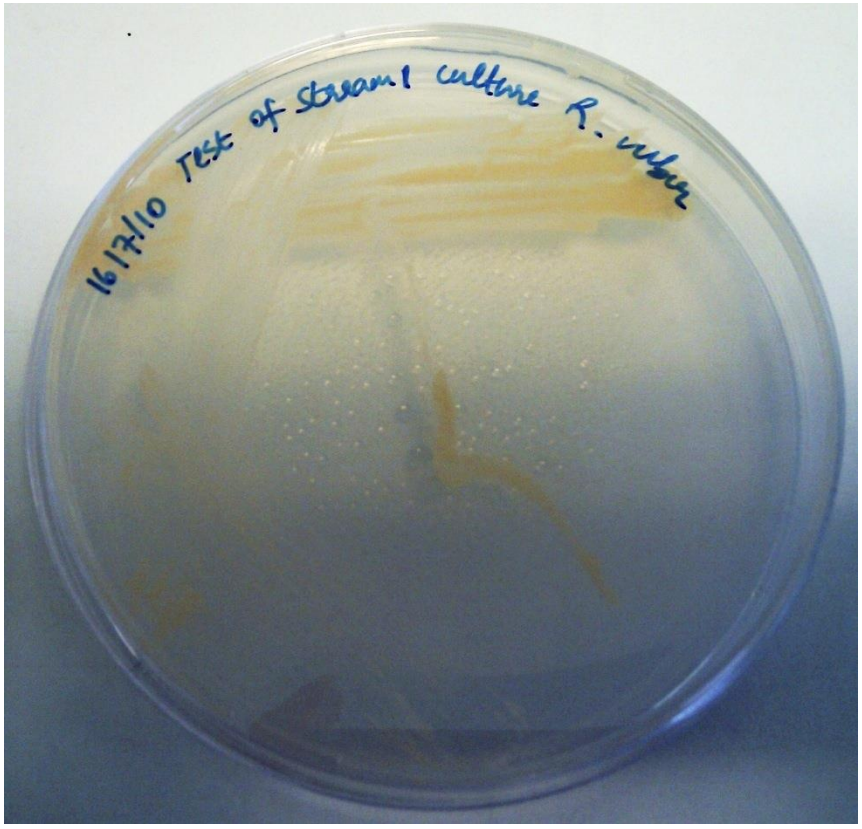


Figure 5.9: Contamination check for *R. ruber* in stream 1

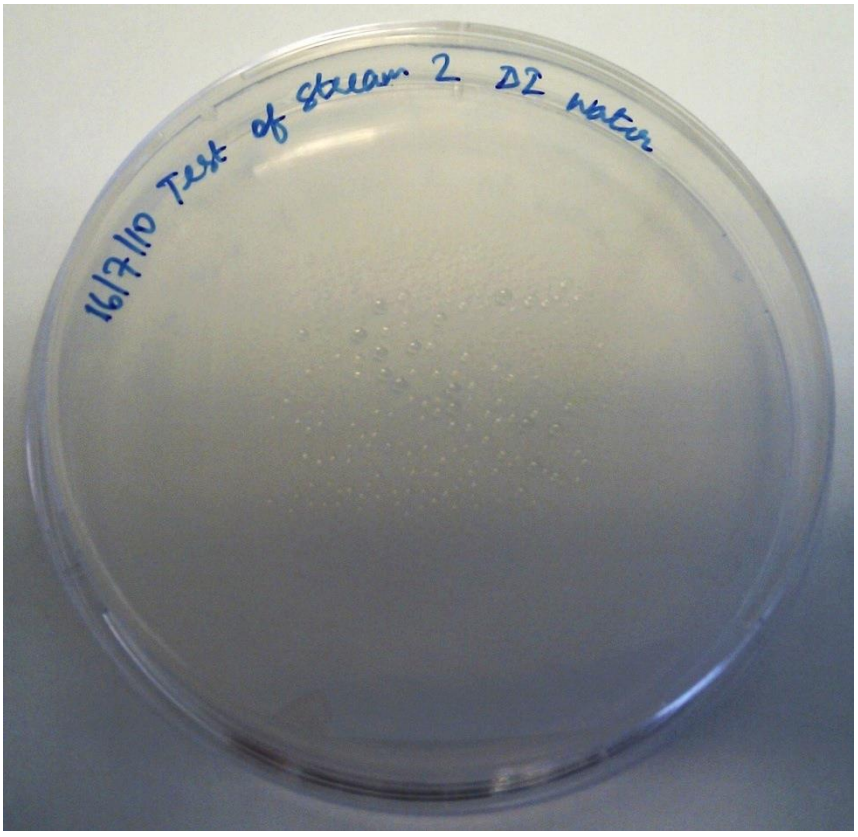


Figure 5.10: Contamination check for stream 2 containing DI water

Table 5.3 below represents first experiment of the membrane reactor that involved the use of *Rhodococcus ruber* grown on glucose (2g/L) in stream 1 and sterilized water in the opposing stream 2. Stream 1 was pH corrected with 2M NaOH solution whereas stream 2 was not corrected. All the values presented below were carried out as duplicate measurements.

Sample ID	**Stream 1**		**Stream 2**	
	OD 600	**pH**	**OD 600**	**pH**
Pre-Sample A	0.075	9.01	0.04	6.96
Pre-Sample B	0.067	8.89	0.07	6.97
Time (hours) 0	0.101	8.57	0.055	6.96
Time (hours) 5	0.107	7.735	-0.0015	7.595
Time (hours) 23	0.164	7.20	-0.0155	7.515
Time (hours) 29	0.172	7.18	-0.0150	7.504
Time (hours) 47	0.462	6.925	-0.014	7.355
Time (hours) 53	0.512	6.93	-0.012	7.32
Time (hours) 71	0.98	6.955	-0.0135	7.255
Time (hours) 77	1.066	6.865	-0.0165	7.385

Table 5.3: Membrane reactor trial with DI water in stream 2

From the readings presented in Table 5.3 above it is evident that there is a drift in the pH of the two streams. The pH in Stream 1 initially was set at pH 9 but towards

the end of the run at 77 hours it had reduced to 6.8 whereas the pH of Stream 2 increased from an initial pH of 7 to approximately 7.4. It is possible that if the experiment were allowed to continue further both the streams would have attained an equilibrium pH. This clearly shows that there is a diffusion of media across the two streams but no exchange of bacterial cells, which is evident from Figure 5.10 that shows that no bacterial cells were present in Stream 2.

The Table 5.4 below represents the data collected during the first run of the membrane reactor while using both the strains of bacteria, which is also represented in Figure 5.11 and Figure 5.12. The experiment was not carried out to completion due to technical difficulties explained in the discussion of results. All the values presented below were carried out as duplicate measurements.

Time (hours)	**Stream 1 *Rhodococcus ruber***		**Stream 2 *Rhodococcus opacus***	
	OD 600	**pH**	**OD 600**	**pH**
Pre-Sample	0.138	7.815	0.088	7.82
0	0.271	7.76	0.29	7.845
4	0.218	7.83	0.262	7.825
23	0.181	7.525	0.194	7.55
47	0.112	7.29	0.210	7.31
97	0.220	7.615	0.262	7.39

Table 5.4: Optical density and pH measurements using membrane reactor

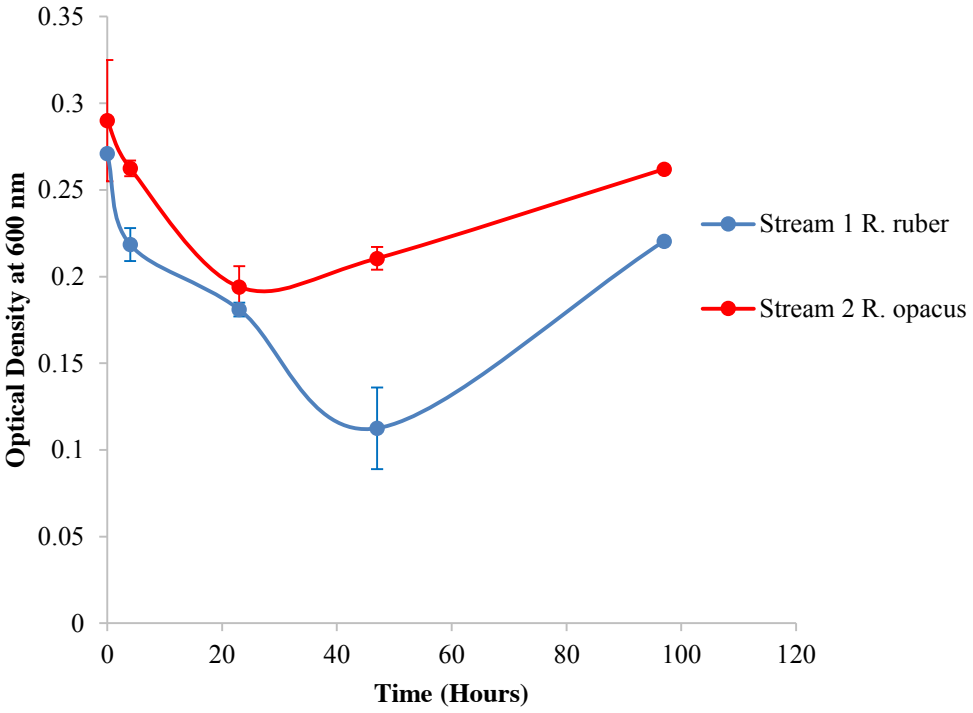


Figure 5.11: Bacterial growth curve for Experiment 1 using membrane reactor

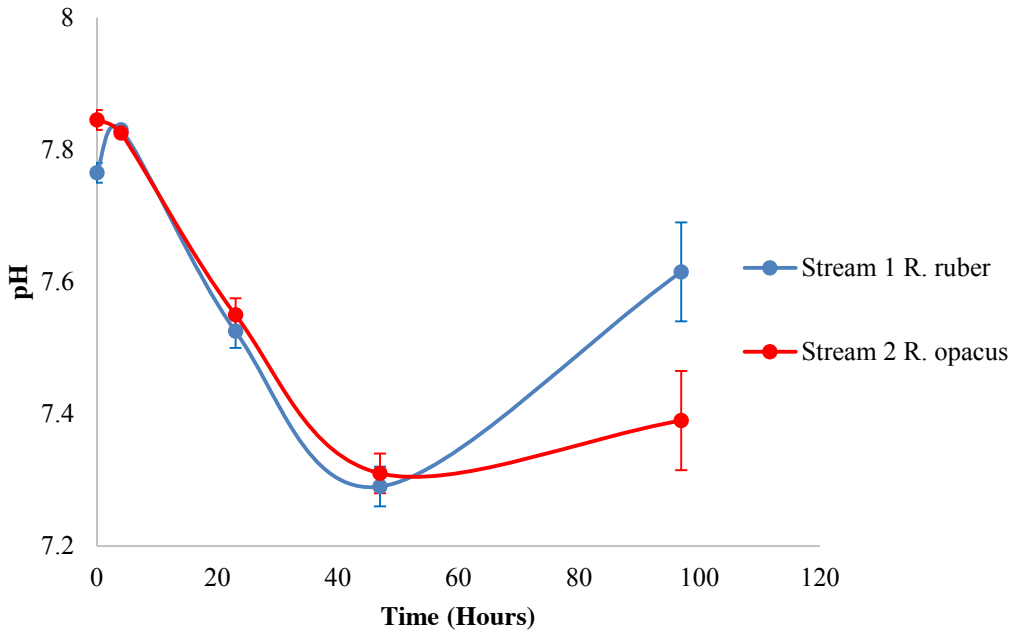


Figure 5.12: pH drift for Experiment 1 using membrane reactor

Time (hours)	Stream 1 *Rhodococcus ruber*		Stream 2 *Rhodococcus opacus*	
	OD 600	**pH**	**OD 600**	**pH**
0	0.27	8.025	0.29	8.235
6	0.16	8.015	0.28	8.100
24	0.27	7.810	0.33	7.725
30	0.42	7.560	0.50	7.415
48	2.16	7.465	1.88	7.78
72	4.54	7.500	2.34	7.425
96	4.78	7.515	2.22	7.42
168	0.94	7.220	1.41	7.165

Table 5.5: Optical density and pH measurements using membrane reactor experiment set 2.

The data represented in Figure 5.13 and Figure 5.14 were plotted using second set of experiments, which were modified to allow for the technical problems that were encountered with the initial run. The modifications are detailed at the end of the chapter.

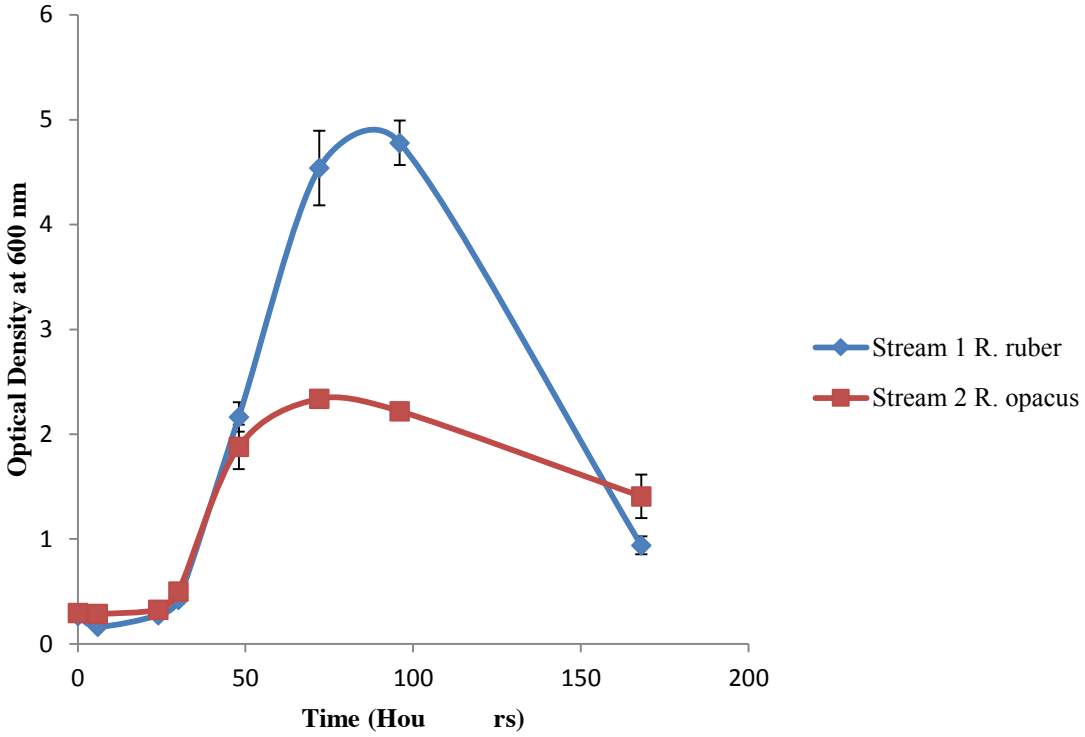


Figure 5.13: Bacterial growth curve for Experiment 2 using membrane reactor

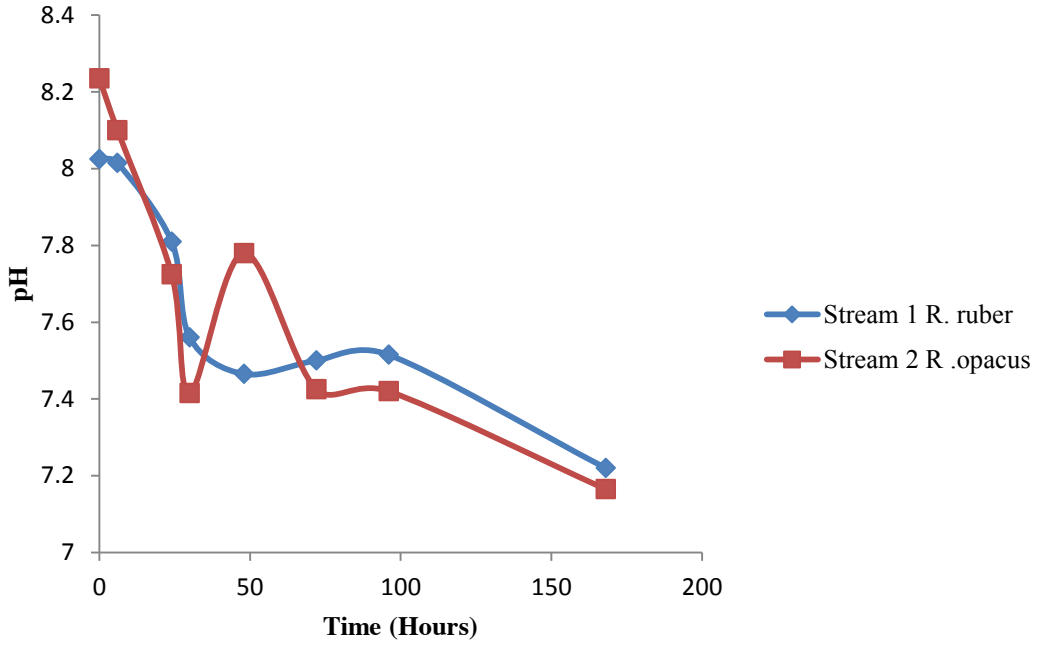


Figure 5.14: pH variation in culture media for Experiment 2 using membrane reactor

Table 5.6 represents the surface tension values obtained when both the bacterial strains were cultivated simultaneously in opposing streams using the membrane reactor. Two sub-samples were collected every time from each feed container and every sub-sample analysed a total of 5 times using a torsion balance for surface tension and interfacial tension (White Electrical Inst. Co. LTD).

	Surface Tension Value (dynes/cm) of *R. ruber*						
Time (Hours)	**A**	**B**	**C**	**D**	**E**	**F**	**Average**
6	63	64	63	65	64.5	66	64.3
30	57.5	59.5	57	58	57	58.5	57.9
48	45	39	42.5	42	41.5	40.5	41.8
96	31.5	32	32.5	31	31.5	33.5	32.0
168	32.5	33	33.5	31.5	35	32	32.9
	Surface Tension (dynes/cm) of *R. opacus*						
Time (Hours)	**A**	**B**	**C**	**D**	**E**	**F**	**Average**
6	64	62.5	64.5	65.5	65	64	64.3
30	59.5	57	58.5	56	59	57.5	57.9
48	43.5	42	44	42.5	43	45	43.3
96	32.5	29	29.5	31.5	32.5	31.5	31.1
168	33	33.5	32.5	34	34.5	33.5	33.5

Table 5.6: Comparison of surface tension values between *R. ruber* and *R. opacus* obtained during cultivation using the membrane reactor

The data represented in Figure 5.15 and Figure 5.16 were plotted using third set of experiments, which were modified to allow for the technical problems that were encountered with the initial run.

Time (hours)	**Stream 1 *Rhodococcus ruber***		**Stream 2 *Rhodococcus opacus***	
	OD 600	**pH**	**OD 600**	**pH**
0	0.2805	8.025	0.2995	8.18
6	0.2295	8.045	0.2495	8.16
24	0.2720	7.97	0.353	8.02
30	0.499	7.815	0.527	7.855
48	2.07	7.42	2.228	7.425
72	4.752	7.355	3.986	7.415
96	5.096	7.41	3.4	7.415
168	1.946	7.315	1.582	7.32

Table 5.7: Optical density and pH measurements using membrane reactor experiment set 3.

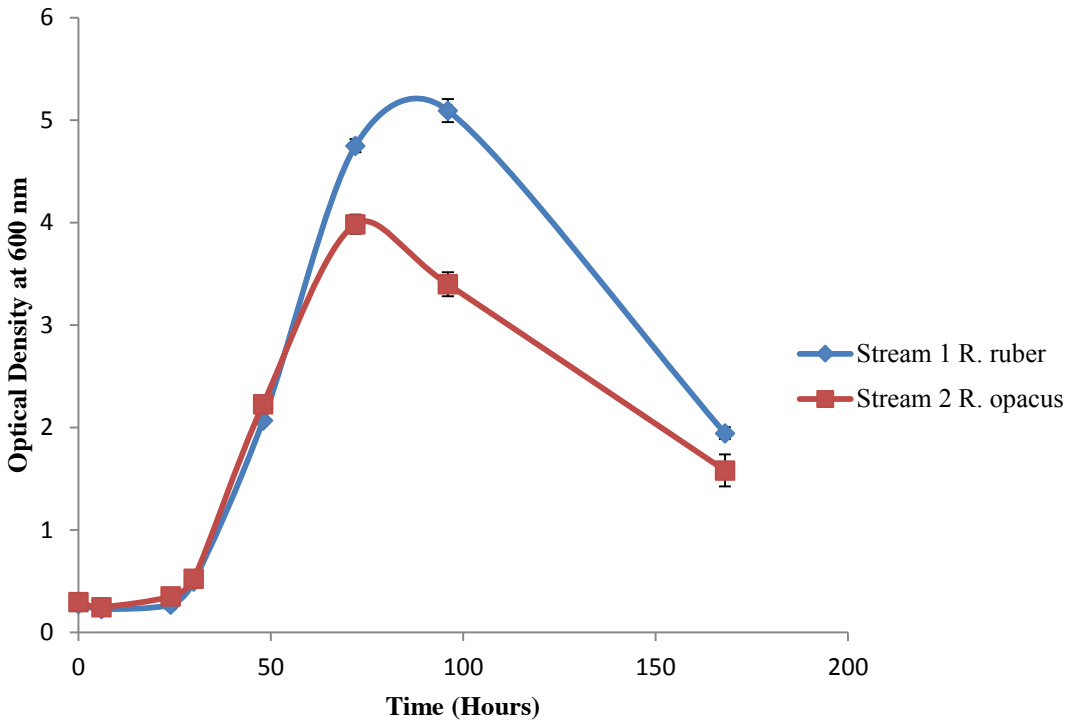


Figure 5.15: Bacterial growth curve for Experiment 3 using membrane reactor

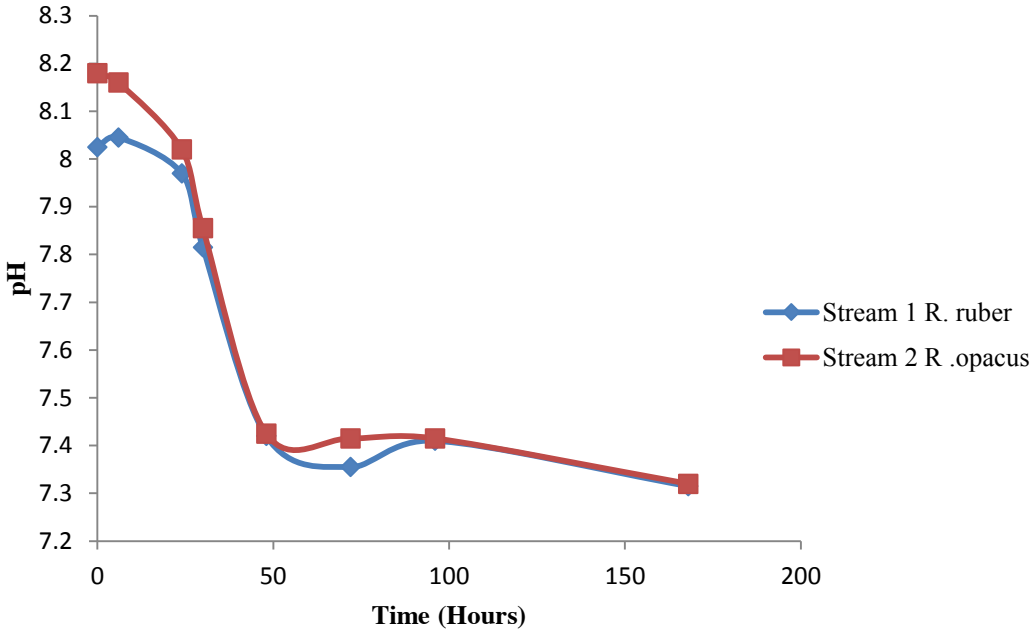


Figure 5.16: pH variation in culture media for Experiment 3 using membrane reactor

The first experiment carried out using the membrane reactor involved the use of *Rhodococcus ruber* grown on glucose as the carbon source while the opposing stream consisted of plain sterilized deionised water. The previous tests have proven that the surfactants are able to pass through the filter membrane while blocking passage of the bacterial cells but the same needed to be tested on the membrane reactor. Due to the construction of the reactor it could possibly allow cells to pass between the individual filter modules that were connected to each other using mechanical elements. Contamination of the feed is another possibility because due to the larger scale of operation, it was not possible to control the growth parameters as rigorously as the flask cultures that were carried out aseptically in fume hoods and closed incubators. At the end of the experiment, the feed from both the streams were inoculated on to solid agar plates and visually examined for contamination as seen in Figure 5.9 and Figure 5.10. This was performed for all the experiments carried out using the membrane reactor. The agar cultures showed no contamination and more importantly there was no transfer of bacterial cells between the individual filter modules.

When the bacterial strains were grown together on the opposing streams, a problem that was prevalent in the previous experiment was still present. When the experiment initially started, because of minor differences in the medium composition due to the addition of the seed inoculum into each feed container, there was an osmotic potential difference. This imbalance led to unequal media diffusion between the filter modules and resulted in either one of the sides eventually depleting of culture media. This was evident on constant monitoring of the level present in the feed containers. In these experiments at regular intervals the feed containers were sub-sampled to allow for growth measurements. It was possible to accurately measure the pH changes and surface tension values but it was not possible to quantitatively measure the optical

density since the feed container was not representative of the actual cells that were present. As observed in Figure 5.8, bacterial cells were present inside the membrane reactor. While this is advantageous overall for the operation of the membrane reactor, it makes quantifying the bacterial cells through optical density measurements difficult and inaccurate. These were the reasons for terminating the first experiment with both bacterial strains prior to completion.

In the subsequent experiments the problems described above were minimised. After the initial setup of the experiment, the level of the culture medium in the feed containers were observed at regular time intervals and the speed of the centrifugal pumps were adjusted to make up for the loss of media at either side of the membrane reactor. The feed containers were sufficiently sealed to prevent loss of media by evaporation and occasionally freshly prepared and sterilized media were added to make up for the loss of media since the experiments were carried out for a longer time period. Also in order to obtain more accurate measurements of growth, during sampling from the feed container the pumps were operated in the reverse direction temporarily to flush media and bacterial cells present in the membrane reactor back into their respective feed container and subsequently normal operation was resumed. This operation is required in order to obtain accurate optical density measurements. Since the media and the bacterial strains are being pumped back into their respective feed or sample containers, the overall integrity of the membrane reactor is still maintained with bacterial cells constrained within their original streams.

Results obtained from the membrane reactor showed that the growth of *Rhodococcus opacus* is better than the growth achieved using the automated batch

reactor. In the second experiment using both the bacterial strains, the stirring speed and the flow in the membrane reactor was increased to allow greater mixing to avoid clumping of cells within the feed container and inside the membrane reactor. While measurements were made to ascertain the surface tension values of the culture media due to the working nature of the membrane reactor, an extra set of sub-sample was collected to minimize errors. Usually three sets of readings are taken on a single sample using the torsion balance but in this experiment two sets of samples were taken and each were analysed three times and an average of 6 readings were taken as the average surface tension value. Comparing results obtained for pH and the surface tension, it is clear that there is good exchange of media within the reactor. The lowered surface tension values were not obtained using *Rhodococcus opacus* on any of the substrates that were used before and can be attributed to the presence of *Rhodococcus ruber* present in the opposing stream.

In spite of the various precautions taken during the course of the experiments conducted using the membrane reactor, it was not possible to establish growth similar to the flask cultures both individually and in mixed cultures. This could possibly be due to the limitations of using a larger working volume and the inability to control the growth parameters more effectively. However from analysing the data that has been obtained from all the experiments conducted, it was evident that the growth of bacterial cells are definitely better when cultivated in the membrane reactor as opposed to when grown together in the batch reactor. This clearly demonstrates that the presence of surfactants is aiding in the enhanced growth of the bacteria, as that is the only difference between the two experimental setups.

Chapter 6: Discussion and Conclusion

This chapter summarises the results that have been obtained in the various experiments conducted during the course of this project. The results obtained from previous chapter concerning the use of mixed culture membrane reactor shows that there is a possibility of expanding this concept into various other applications, which will be discussed in more detail. Finally this chapter deals with the potential future work that could be carried out in the area of mixed culture membrane reactors.

6.1 Discussion of Results and Conclusions:

6.1.1 Chapter 2: Growth Studies and Media Adaptation Conclusions

From the solvent studies carried out in Chapter 2 it can be inferred that *Rhodococcus opacus* could be easily adapted to grow on a number of different solvents. Even though different sources of carbon were used, a clear distinction could be observed in the growth pattern. Hydrophobic and hydrophilic carbon sources elicited different behaviour from both the bacterial strains. For example, in water-soluble sources such as methanol, glucose and glycerol, the bacterial cells exhibited no clumping and surfactant levels were muted. Whereas when grown in presence of n-hexadecane the bacterial cell clumping was pronounced and growth was primarily focused on the medium-n-hexadecane interface. Increased surfactant production levels always accompanied this and the clumping effect could only be reduced by using much higher rotation speeds or by using baffled Erlenmeyer culture flasks. As discussed in Chapter 5, this could be a future consideration when designing the membrane reactor as the inability to increase the pumping speeds in order to preserve the membrane integrity could result in cells clumping together.

The observed differences in growth mechanisms when grown in the presence of hydrophilic and hydrophobic carbon sources have an impact on the potential use in a membrane reactor. The growth studies on glucose, methanol, glycerol, crude glycerol and n-hexadecane indicate that the *Rhodococcus opacus* strain is robust and versatile, which is the intended application in a mixed culture reactor.

6.1.2 Chapter 3: Optimisation of Growth Parameters Conclusions

Another observation was the marked difference in the growth patterns when cultivated at different temperatures. Relatively small temperature differences between 2ºC to 3ºC had a marked impact on the overall growth, which is directly linked to the surfactant levels present in the growth media. As discussed in the literature review this phenomenon further illustrates the different nutrient uptake mechanisms and ability to rapidly adapt to changing growth conditions.

Optimisation was carried out on a limited scale as complete parameter optimisation is beyond the scope of this project. This was carried out more to identify the level of adaptability of the two strains of bacteria. Nitrogen source optimisation was a key area because often changes in the source produced drastic changes in surfactant expression levels and also the structure of the molecules. This phenomenon has been observed and documented in experiments that were carried out much earlier by other researchers (Geurra-Santos *et al*., 1984) and considerable growth differences were found in experiments carried out in this project. From the results obtained, it can be shown that the bacteria have a positive growth response in the presence of nitrate salts, which were not present in the presence of ammonium salts. Further observations using separate set of experiments revealed that the presence of ammonium salts caused a drop in the pH of the growth media, which is not observed in the presence of nitrate salts. On a separate note, in certain experiments carried out in various pH ranges it was clearly

evident that *R*. *opacus* could easily adapt to pH changes hence to a certain extent negating the above said preference to nitrates as illustrated in Figure 3.7 and Figure 3.8. This would be advantageous in certain operating conditions such as when the other chosen bacterial strain in the co-culture shows enhanced growth potential in ammonium salts.

Another important growth parameter tested included the presence of NaCl in the culture media. *R*. *opacus* was grown in varying salt concentration from 1g/L to 12 g/L and this had negligible effect on the actual growth capability as illustrated by Figure 3.5. Considering that typical salinity of seawater is 3% to 4% this could be hugely advantageous in future work on this strain. The ability to not only survive but not be affected by such high NaCl concentrations would make *Rhodococcus opacus* ideally suited for in-situ bioremediation where the use of other normal bacterial strains would be not possible due to the hostile growth environment.

Translated to real world applications, this would mean that when used in a membrane reactor, the medium could potentially be tweaked to enhance growth of any other bacterial culture used in the membrane reactor since *Rhodococcus opacus* could be adapted to survive in varied growth conditions.

Overall based on the results obtained from nitrogen source optimisation experiments shows that media optimisation plays a key role in biosurfactant production levels which supported by similar research that has been carried out (Moussa *et al*., 2006; Abouseoud *et al*., 2008). Results from the sodium chloride experiments as illustration in Figure 3.5 and Figure 3.6 show that *Rhodococcus opacus* can survive at high concentrations, which would be beneficial for in-situ applications. Comparing results illustrated in Figure 3.3 and Figure 3.4 show that *Rhodococcus opacus* is not affected by a wide range of pH whereas *Rhodococcus ruber* was found to be less robust.

This would have an implication in the design of the mixed culture reactor and the pH would need to be monitored regularly to ensure that the pH conditions are maintained to obtain optimum levels of *Rhodococcus ruber* growth, which is directly linked to surfactant production levels.

6.1.3 Chapter 4: Biosurfactant Characterisation Conclusions

In order to be used in future experiments and to classify the surfactants that were being expressed by the two bacterial strains further experiments were carried out. During the course of these experiments the difference in the mechanism of carbon uptake between the two strains were further observed. Surfactant expression dramatically increased in the presence of hydrophilic carbon source and as discussed in Chapter 4, *Rhodococcus opacus* was found to produce surfactants that were bound to the cell wall whereas *Rhodococcus ruber* had a tendency to produce surfactants that were primarily released into the culture media as presented in Table 4.3.

This is evident after observing the results obtained in both the emulsification index values as illustrated by Figure 4.1, Figure 4.2 and Figure 4.3 and the BATH assay results represented by Figure 4.4 and Figure 4.5. This clearly indicates a difference in the carbon uptake mechanism. In the case of *R. opacus* since the surfactants are bound to the cell wall the uptake could either be through direct contact with the hydrocarbon by growing on the hydrocarbon-medium interface or if there is sufficient mixing to create contact. On the other hand with *R. ruber* the mixing parameter need not be as efficient since this is compensated by the production of surfactants that are release into the medium thus facilitating the carbon uptake.

This would have an impact on the type of role these two strains could be utilized for when grown in a mixed culture membrane reactor. Considering this difference in the uptake mechanism indicates that R. ruber would be the better candidate to use in future

experiments where one of the bacterial strains were chosen for their ability to produce surfactants that are released into the culture media and thus reduce the surface tension of the medium. The results concerning the surface tension values indicate that both the bacterial strains are promising candidates for use in future work. Both the bacterial strains were capable of considerably reducing the surface tension of the medium that they were cultivated in. For instance even the use of a crude surfactant extract obtained from the culture media used to cultivate *Rhodococcus ruber* was capable of reducing the surface tension of freshly prepared media to 30 dynes/cm. This is a significant achievement when considering that the extract used was not purified and potentially contained solvent impurities that were carried over during the solvent extraction procedure.

To summarise, results from Table 4.1, Table 4.2 show that surfactants are cell bound on *Rhodococcus opacus*, which also corresponds to results obtained in Chapter 2 where a clear difference existed between cultures grown on hydrophilic and hydrophobic carbons sources. Research carried out by Ciapina (2006) proved that surfactants were indeed cell bound on certain bacterial species associated with biosurfactant production. Results from Table 4.3 show that *Rhodococcus ruber* would be an ideal candidate in a membrane reactor due to the fact that as opposed *R.opacus* this bacterial strain releases biosurfactants into the growth media which would in essence aid other microorganisms in hydrocarbon uptake due to the reduced surface tension. Another important characteristic of this bacterial strain is presented in Table 4.6 and Table 4.7 which show that in spite of not producing significant quantities of surfactant in the presence of hydrophilic carbon sources, *Rhodococcus ruber* was capable of significantly lowering surface tension in the presence of a hydrophobic carbon source. This is greatly beneficial in a membrane reactor.

6.1.4 Chapter 5: Mixed Culture Reactor Design and Operation Conclusions

Studies have indicated that use of surfactants enhance the degradation capabilities of organisms. However this is usually carried out by adding purified surfactants of either chemical or biological origin into the culture broth, which would add to the overall cost of the procedure (Van Hamme & Ward, 2001, Rahman *et al*., 2003, Karpenko *et al*., 2004, Haddadin *et al*., 2008, Kolomytseva *et al*., 2008). Trejo-Castillo et al. (2013) showed that the addition of crude biosurfactant mixture extracted from a mixed culture of 7 bacterial strains in a 1.4 L tubular reactor enhanced biodegradation of hydrocarbon contaminated soil slurry. Their findings showed that the addition of surfactant as a pre-treatment decreased the hydrocarbon content from 9275 g/Kg to 674mg/Kg, which represents 92% removal efficiency. The same study also showed that adding the surfactants during inoculation but not as a pre-treatment reduces hydrocarbon content only by 34%.

In another similar research work carried out by Lin Qin et al. (2012) further validates the results obtained in this project. Lin Qin et al. (2012) studied the effect of the presence of additional Rhamnolipid in a novel submerged membrane reactor. This study looked at using Rhamnolipid produced by *Pseudomonas spp*. Zju.um1 to enhance degradation of waste frying oil. Their research showed that the addition of Rhamnolipid increased the oil removal efficiency by 90% and exhibited 10 times higher membrane permeability when compared to the control reactor. The use of the surfactant also prevented membrane fouling due to the fact that the oil droplets strongly adhere to the surface of the flocs and consequently prevents the large oil droplets from directly depositing on the membrane surface. Recent research carried out by Vanessa et al. (2011) show that mixed bacterial cultures are extremely effective at degrading aliphatic

and aromatic hydrocarbons. This study looked at a consortium of bacteria that included *Stenotrophomonas acidaminiphila*, *Bacillus megaterium*, *Bacillus cibi*, *Pseudomonas aeruginosa* and *Bacillus cereus* to degrade oily sludge when cultivated for 40 days as sole carbon source. Results show that the mixed culture was able to reduce 90.7% of the aliphatic fraction and 51.8% aromatic faction as well as reducing surface tension by 39.4%. Results from experiments carried out in this project compare very favourably with these results where *Rhodococcus ruber* was able to achieve 58% reduction in surface tension values.

Instead as discussed above if this could be replaced by the use of live bacterial strain like *Rhodococcus ruber* in a separated membrane reactor then this would be beneficial cost wise and also more practical in real world applications because the bacterial cultures could be re-used by having a semi-continuous mode of operation. Another reason for this method being more economical is that with the use of a membrane reactor the downstream processes utilized to extract the surfactant would not be required. This would be more beneficial than bio-augmentation (Akio *et al*., 2007) where exogenous bacteria/bacterial systems are added into the contaminated site primarily because of environmental issues that might have to be considered. Another disadvantage with bio-augmentation is that once the bacterial consortium is introduced into the contaminated site there is no absolute way of quantifying either the growth or effectiveness due to external factors that come into play. All of these obstacles could be avoided with the use of larger scale membrane reactors, which could be potentially run with negligible interference from the nature of contaminants and the bacteria being utilized.

As discussed in Chapter 5, the results obtained from all the experiments that were carried out using the two bacterial strains were utilized while conducting mixed culture experiments. One of the key criteria to be considered for optimum working of

the reactor is the membrane selection as the separation potential solely depends on the nature of the membrane being utilized. Since the work carried out in this project and other potential applications involve the use of hydrocarbons, the chosen membrane was required to not be susceptible to hydrophilic substrates. The various reasons for using a membrane reactor become apparent when data from previous experiments involving growth potential of the bacterial strains were compared.

The growth of the bacterial strains individually has been consistently better than when cultivated together. This has had implications in this project as well as other work being carried out that involves the use of bacterial consortiums. Even though maximum growth potential was not achieved when the bacterial cells were grown together, the same effect has not been observed when *Rhodococcus opacus* was cultivated in the presence of surfactant extracted from *Rhodococcus ruber*. On the contrary, the growth has in fact been enhanced due to the presence of surfactant from another bacterial species. This clearly indicates that even though the direct bacterial co-existence may be antagonistic to growth, the presence of surfactants from one species appears to have a positive impact on growth. As mentioned previously, the use of bacterial consortium in bioremediation has been proven to be better than individual bacterial strains in certain cases but that need not always be the situation as shown in this project. However with the use of membrane reactors, this factor need not be considered at all. Bacterial strains need not be tested prior to being used in an experiment, which means that this method could prove to be more robust and faster to adapt based on the circumstance.

This is the principle that is being exploited with the use of membrane reactor in this study. The effectiveness in the use of the membrane reactor can be understood by comparing growth data from experiments carried out using the membrane reactor and those using *R*. *opacus* grown in the presence of surfactant extracted from *R*. *ruber*. As demonstrated in the previous chapter, the growth of R. *opacus* was increased in the

presence of surfactants and was directly linked to the concentration of the surfactant as shown in the graph in Figure 5.4. This is coupled with the data that showed that the growth in the membrane reactor was more pronounced than in the automated batch reactor when the bacterial species were cultivated together. This was despite having problems with the smooth operation of the membrane reactor. In addition, the data obtained from mixed culture flask experiments also mirrored growth pattern obtained using the batch vessel. Similar research carried out by Olivia et al. (2012) compared the different mechanisms of hexadecane uptake among pure and mixed cultures. Their study involved four organisms *Xanthomonas spp*., *Acinetobacter bouvetti*, *Shewanella spp*. and *Defluvibacter lusatiensis*. Results showed hat the organisms exhibited different combinations of uptake mechanisms such as direct contact and biosurfactant-mediated uptake. They also discovered that the mixed cultures showed enhanced n-hexadecane removal when compared to pure cultures.

In a similar study to those experiments carried out in this project, Francois Coutte et al. (2013) found that the use of bubbleless membrane bioreactor (BMBR) as beneficial in the extraction of surfactin from Bacillus subtilis BBG131 strain. The study found that using a membrane reactor enhanced the biosurfactant productions as compared to a batch reactor, which did not offer the same levels of flexibility and produced less than half the surfactant (17.4mg/L/hr versus 54.75mg/L/hr). The research also shows that productivity can be improved by continuous production, extraction and purification by using a integrated bioprocess efficiently in smaller reactor volumes.

From all the data obtained during the course of this project, it is possible to conclude that the use of mixed culture membrane reactors are definitely promising especially in the field of bioremediation.

6.2 Summary:

The overall advantages of using a mixed-culture membrane can be summarized as follows:

1. The key point outlining the advantage of mixed-culture membrane reactors is demonstrated in the experiments detailed in Chapter 5, where results from three different experimental setups were compared. Bacteria were cultured individually, as a mixed culture grown together and finally using the membrane reactor. Among all the methods, the growth observed in the membrane reactor clearly surpasses growth observed when grown together in mixed culture. This indicates that the method can be modified to suit the situational requirements and could be used with entirely different set of bacterial strains.

2. Membrane reactors would reduce the overall cost of the operation as traditionally chemical or biological surfactants were added to culture broth to enhance bio-degradation. However with the use of membrane reactors, they could be grown along side without the need for expensive downstream processes to purify and extract surfactants.

3. In areas where the introduction of exogenous bacteria is not feasible due to either extreme environmental conditions or legal issues, mixed-culture membrane reactors can be easily adapted and utilized for bioremediation instead.

4. In certain environmental conditions where the efficacy of a biological agent needs to be determined, it would be difficult to do so in-situ once the bacteria have been introduced to the contaminated site. Use of membrane reactors would give complete control and online measurements of all the growth parameters.

6.3 Future Work:

Some of the ideas mentioned in this work can be further explored in order to gain an understanding of the underlying principles. Hence future work needs to concentrate on the following aspects in order to gain a better understanding of the nature of surfactants in general and how they interact depending on the growth parameters.

1. The structure and characteristics of the surfactant molecule needs identifying and investigation of change in the structure of the molecule with different changing growth conditions should be carried out.

2. Monitoring of the optical density and growth on-line in real time would be advantageous to gain a clear understanding of how exactly growth and surfactant expression levels are related.

3. As seen in the previous chapters, minor changes in reactor/growth parameters such as temperature, mixing speeds and pH have an effect on the overall growth. Having observed this effect, more complex optimisation of growth parameters need to be carried in order to achieve maximum surfactant expression.

4. Another phenomenon observed in the membrane reactor that needs to be addressed is the clumping of bacterial cells. From the work that has been carried out in this project it can be inferred that this is due to difference in carbon uptake mechanism. For efficient utilization of the carbon source there needs to be sufficient mixing but this is a limiting factor since membrane reactors can only be operated under certain pumping speeds as dictated by the membrane being used. This could be overcome by potentially using built in baffles inside the individual membrane modules thus overcoming the above problem.

5. Metabolic growth modelling has not been carried out for the two bacterial strains that were utilized in this work. Experimental modelling when coupled with kinetic modelling could have a positive impact on gaining an insight of how bacterial strains would react to the altering of various growth parameters as seen by Shen *et al*. (2008).

6. As discussed in earlier chapters, results prove that the presence of surfactants isolated from *Rhodococcus ruber* improved the growth of *Rhodococcus opacus* (Figure 5.4). Due to the working nature of this reactor, another way this reactor could be exploited is by using a surfactant-producing bacterium in one stream and to use a co-culture bacterial system in the other stream, which has proven degradative capabilities. This could potentially be applied to any mixed culture bacterial systems.

7. Finally more work needs to be done to analyse secondary metabolites produced in order to map hydrocarbon uptake. This would give a better understanding of the uptake mechanism and the reason for the difference in the growth observed when grown on varying nitrogen sources.

8. It could be beneficial to investigate a continuous mode of operation as shown by Francois Coutte et al. (2013), which proved that a continuous operation would enhance the biosurfactant production levels. Another study that could be investigated is the use of crude surfactant extracted from Rhodococcus ruber to pre-treat hydrocarbon contaminated samples as show by research carried out by Trejo-Castillo et al. (2013).

Appendix A

Medium B Composition:

Constituent	Quantity per Litre of Deionised water
K_2HPO_4	0.8g
KH_2PO_4	0.2g
$CaSO_4.2H_2O$	0.05g
$MgSO_4.7H_2O$	0.5g
$FeSO_4.7H_2O$	0.01g
$(NH_4)_2SO4$	1g

SL-6 Composition:

Constituent	Quantity per Litre of Deionised water
ZnSO4.7H2O	0.1g
MnCl2.4H2O	0.03g
H3Bo3	0.3g
CoCl2.6H20	0.2g
CuCl2.2H2O	0.01g
NiCl2.6H2O	0.02g
Na2MoO4.2H2O	0.03g

BATH Assay Composition:

Constituent	Quantity per Litre of Deionised water
K_2PO_4	16.9g
KH_2PO_4	7.3g
Urea	1.8g
$MgSO_4.7H_2O$	0.2g

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